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Physiological Studies in Plant Nutrition

XVI. The Mineral Nutrition of Bracken

Part I. Prothallial Culture and the Effects of Phosphorus and Potassium Supply on Leaf Production in the Sporophyte[1]

BY

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With Plate XVIII and four Figures in the Text

Abstract

Part I of this paper describes culture methods for the production of large numbers of bracken sporelings of uniform size. Observations on the growth of the bracken plant and results of experiments on the mineral nutrition of the prothallial and sporophyte generations are presented. The presentation of further growth data and a full analysis of the results is deferred to the second part of this paper.

[1] Part of thesis submitted to the University of London for the Ph.D. degree in 1949. This investigation was supported by a grant from the Agricultural Research Council.

Introduction

In recent years bracken has become important as a weed of agricultural land, especially in the upland districts of Scotland and Wales. Changed agricultural practices are held to be the cause favouring its spread (Braid, 1934). As an aid to the understanding of the advance of existing bracken colonies and the rarer event of the establishment of new centres from prothalli, a general investigation into the basic inorganic nutrient requirements of the bracken plant was considered desirable, even though it was not designed to yield results directly applicable in its control. Extension of the methods of growth analysis to the mineral nutrition of a cryptogam was also considered of interest as an indication of the extent to which conclusions drawn from past work at this Institute with phanerogams might be regarded as generally applicable.

The only nutritional experiments with ferns appear to be those of Czaja (1921), Prantl (1881), and Nagai (1914), on the prothallial generation; not a single paper on the nutrition of the diploid generation of any fern was found.

Propagation of the necessary plant material proved to be somewhat difficult and some considerable time was spent during this investigation in finding a satisfactory method of raising large numbers of plants of uniform size on which successful growth experiments must depend. None of the methods of raising the prothallial generation described in the literature (Lagerberg, 1907; Twiss, 1910; Lawton, 1936, &c.) were suitable since they were generally designed to produce only relatively small numbers of plants without aiming at uniformity of size. Hence part of this paper will be devoted to a description of the culture methods and of a few nutritional experiments on the prothallial generation. This will be followed by a description of the design and methods of the main experiment on the nutrition of the sporophyte. The results have been divided into two groups: those of a more ecological interest will be presented below, while the main growth data will be presented in a second part.

Culture Methods

Unfortunately there is no stage in the pteridophyte life-history which is comparable to the phanerogam seed in its convenience for raising a new plant. The two methods of raising the necessary plant material which suggested themselves were: (1) vegetative propagation of short lengths of rhizome collected from existing colonies, and (2) raising of young sporelings from spores.

The first method has the advantage of working with a clone; it would seem that uniformity of plant size might be achieved by careful selection of the lengths of rhizome. However, this method suffers from the serious disadvantage that by its use one is confined to plant material of unknown nutritional history. Further, preliminary experiments have shown that regeneration of isolated pieces of rhizome is much more difficult in bracken

than in some other ferns. Pieces at least 6 in. in length with buds, including at least one leaf-bud, are required for any regeneration to take place at all. Such cuttings, taken in April, produced only 1–2 leaves in the first year, so that two seasons are needed before the plant is fully established.

Propagation from spores completely eliminates these disadvantages, and by suitable care in the collection of spores—such as using only spores from one plant or even one sporophyll—genetical variability may be reduced.

Collection of spores, their surface sterilization and method of sowing

Preliminary experiments have shown that it is essential to obtain spore material which is free from the admixture of leaf debris and sporangia. Spores germinating inside the sporangial case generally develop into clusters of prothalli more or less restricted in their growth by active competition. Moreover, the leaf debris, and especially the sporangia, proved to be sources of heavy infection with fungal and other organisms which were frequently parasitic on the prothalli. Infections of this type were so abundant and destructive (generally destroying every prothallus in a culture dish in a very short time) that the successful raising of large numbers of young plants necessitated some preliminary treatment to reduce contaminants.

The method of spore collection finally employed materially reduced these disadvantages. A single mature sporophyll is placed between two sheets of paper and allowed to dry. The spores shed when the sporangia dehisce are practically pure and free from sporangial cases and leaf debris. Even this material is not completely free from hostile contaminants. Since one leaf can yield sometimes as much as 1 g. of spores, all those necessary for an experiment may easily be derived from a single frond of the parent plant. In order further to eliminate all parasitic fungi a few unsuccessful experiments were made to surface sterilize the spores themselves using chemical methods applicable to some kinds of biological material. Concentrated sulphuric acid —frequently used for seed sterilization—partly dissolved the spores within a few seconds, and alcohol by itself or an alcoholic solution of mercuric chloride, if effective in destroying the contaminants, also killed practically all the bracken spores.

Aqueous mercuric chloride was tested in various concentrations and 0·036 per cent. was found to be effective without actually killing the bracken spores, yet their growth was retarded for a long period. Calcium hypochlorite giving a solution of about 1 per cent. Cl_2 (Wilson, 1915) was found to be lethal. However, Knudson (1940) has successfully used a lower concentration of calcium hypochlorite for the sterilization of fern spores. During these tests it was found that merely washing the spores repeatedly with sterile water was sufficient to eliminate nearly all contaminants. Centrifugation between the washes also served to remove light spores and occasional empty sporangia containing air-bubbles. Ten to twelve washes resulted in practically clean spores. As this method was easy and sufficiently effective it was adopted for the main experiment.

In the first few experiments sowing was effected by dusting with the dry spores, which resulted in usually rather dense but fairly even cultures. With the need for washing the spores this method had to be replaced by one suitable for wet material. A number of different techniques were tried; the use of a small atomizer to spray the suspension on the culture medium proved to be the most successful in ease of application, and a very even distribution of spores was obtained.

Substrates, culture media, and necessity of transplanting

It is evident that optimal cultural conditions will differ very largely at different stages of development of the bracken plant, owing to the minute size of the spore (it was found that 1 mg. contains about 2×10^6 of them) and the very considerable size of the mature plant (single leaves of which may exceed 10 ft. in length). Although in most experiments described below the plants were grown for only 1 year or less, during which time the largest attained about 35–40 g. dry weight, culture conditions needed to be adjusted repeatedly to suit their progressively changing requirements, particularly with regard to light and humidity. Generally the demand for light increases with age, that for high humidity falls.

In all prothallial and the earliest sporeling stages, when danger of fungal attack is greatest, the cultures were subdivided among a large number of small vessels which permitted elimination of contaminants with the minimum loss of plant material. During the whole period until the final planting out a rigorous selection of the material for uniformity was carried out, an obvious necessity since, apart from differences in prothallial size (which incidentally seems to have some effect on the vigour of the sporeling produced), the time of fertilization varies and further irregularities accumulate as the sporelings develop. All these irregularities need to be countered by selection. In the main experiment of the 1946 season more than nine out of every ten plants were finally rejected.

In all the experiments on cultural conditions the artificial media were prepared with a standard mineral nutrient solution, whose composition is shown in Table I.

TABLE I

Standard Nutrient Solution employed for culturing Prothalli and Young Sporelings

$NaNO_3$	0·456 g. per litre	Minor elements given:	
$Na_2HPO_4.12H_2O$	0·150 ,, ,,	B	0·5 mg. per litre
$Ca(NO_3)_2$	0·440 ,, ,,	Mn	0·5 ,, ,,
$CaH_4(PO_4)_2.H_2O$	0·053 ,, ,,	Zn	0·05 ,, ,,
$CaCl_2.6H_2O$	0·111 ,, ,,	Cu	0·02 ,, ,,
$MgSO_4.7H_2O$	0·125 ,, ,,	Mo	0·05 ,, ,,
K_2SO_4	0·185 ,, ,,		

Iron was added as $FeCl_3$, 1 mg. per litre, and also sprayed on the plants from an atomizer in the form of iron citrate, 0·5 mg. per litre, whenever signs of iron chlorosis appeared.

This particular solution was chosen as being intermediate in type between two of the three solutions employed in the main nutritional experiment.

(i) *Young prothalli.* A number of different substrates and culture vessels for raising the young prothalli through their first stages were tested. The use of seed-germinating dishes containing mineral nutrient agar was finally adopted. The dishes were made of green glass and the centres of their hemispherical lids drawn out into an open neck, which was plugged with cotton-wool; through it the cultures could be watered readily without admitting air-borne contamination.

(ii) *Mature prothalli.* When the prothalli had attained a diameter of about 2 mm. they were transplanted into sand culture. Crystallizing dishes covered with Petri-dish lids were used as culture vessels. Together with the sand-culture solution, they were autoclaved before use to prevent fungal contamination as well as algal growth. Algae—especially members of the Cyanophyceae—can be very harmful, partly owing to their shading effect when growing over the prothallial surface, but chemical (perhaps antibiotic) factors may also play some part. A sterile wire with flattened end was used to transfer the young prothalli from the agar to the saturated sand. The plants suffered practically no check and continued to grow rapidly. The cultures were watered with sterile water and remained remarkably free from hostile organisms.

(iii) *First leaf sporelings.* When the prothalli had reached a size of 8–10 mm. diameter and archegonia and antheridia had been developed, it was found possible to synchronize fertilization to some considerable extent, and thus also the production of young sporelings, by regulating the water-supply. The dishes were watered at first only sufficiently to keep the sand moist and no free water was allowed to reach the under-surfaces of the prothalli. After about a week of this treatment the dishes were flooded to such an extent that all prothalli were in contact with liquid water for a short time. This method was very largely successful in inducing the simultaneous advent of a large number of sporelings. The young sporelings were left in these dishes, and thus in a saturated atmosphere, until their primary leaves were fully expanded and the second leaf had started to develop.

(iv) *Pre-rhizome sporelings.* At this stage the prothalli with their sporelings were transplanted again into 3-in. glass flower-pots containing sand saturated with the same standard nutrient solution, each vessel receiving three plantlets. Plants raised up to this stage in a nearly saturated atmosphere are extremely sensitive to dry conditions and gradual hardening was found to be essential. The plants were kept in an unheated greenhouse with shaded windows, and the pots covered with muslin moistened by periodical spraying with water. Gradual reduction in the amount of spraying, followed just over a week later by removal of the muslin, completed the hardening. In view of the great sensitivity of the young sporelings to drought, the method was remarkably successful, losses amounting to less than 2 per cent. Subsequently the plants were grown for several weeks in these pots and after developing 4–5 leaves

on the average began to produce their rhizomes. During this time size differences appeared and tended to increase with age. This was countered by thinning the pots down, first to two and later to one plant each.

(v) *First-year sporophyte* (*'adolescent'*). At this stage, having initiated their rhizome, the young sporophytes were transplanted singly into the large glazed pots (holding 30 lb. of sand) used for the main nutritional experiments.

The three transplantations between the initial sowing and the final sand culture proved fully effective in eliminating severe competition between the plants and permitted them to grow freely during all stages, while selection for uniformity on these occasions materially reduced the somewhat considerable variability of the bracken prothalli and sporelings.

(vi) *Adult sporophytes.* Bracken plants have been successfully grown for 2 or more years in the large glazed pots, but they tend to get rather pot-bound after the first year. Sporophylls were produced from the second season onwards.

In concluding this description of the sand-culture technique as applied to the growth of bracken a few salient dates and figures are presented for the 1946 season to indicate the times occupied by the different stages and the number of plants required to produce finally a moderately uniform population.

Date.	Description.	Numbers.
December 12, 1945	Sowing of spores	—
December 20–1, 1945	Germination complete	—
February 11–14, 1946	Transplanting of prothalli	4,000–4,500
March 18–20, 1946	Culture dishes flooded to induce fertilization	—
April 8–10, 1946	Majority of sporelings emerged	—
April 17–18, 1946	Transplanting of first-leaf sporelings	1,200
June 7–8, 1946	Planting out of established sporelings	400

Water culture

The possibility of raising bracken sporophytes in water culture has also been investigated, and plants have been successfully grown from spores and carried through two generations by this method. Losses from fungal infection have been greater than with sand-culture methods, but growth in general is little inferior.

Dry spores may be sown on the surface of the nutrient and the prothalli grown as floating culture. As long as their upper surfaces are not wetted the prothalli float readily and no external support is required until the advent of the young sporelings. Waxed cork rings or silver wire loops have been used as supports during the early sporeling stages. Later floats of wide glass tubing were employed to support large plants in 10-in. glazed pots.

The rhizomes themselves, though submerged in the solution, which was not artificially aerated, grew perfectly satisfactorily. While the sporophytes are still comparatively small the main practical difficulty lies in shading the solution surface sufficiently to prevent the young fronds unfolding until they have emerged into the air. Cork granules floating on the surface have been used for this purpose with some success. At a later stage the canopy of older

fronds provides sufficient shade by itself. Again sporophyll production has been observed in the second growth season, the plants having been kept in water culture throughout.

Some Observations on the Growth of the Bracken Plant

Before describing the design and results of the nutritional experiments it appears worth while to mention some observations on the growth habit of the bracken plant.

Prothallial generation. Moisture requirements. As has been pointed out above, a moist environment is essential for the germination and growth of the young bracken prothallus. In a preliminary experiment some boxes containing soil or bracken mould were exposed for some months under sporing fronds in the open. Only a few minute and extremely short-lived prothalli were seen, with the help of a hand lens. Similar boxes covered with a glass sheet and transferred to the greenhouse after 8 weeks' exposure would give rise to thousands of prothalli. This factor has also been investigated by Conway (1949), who claims that normal germination takes place on agar cultures in an atmosphere of as little as 15·5 per cent. relative humidity. It appears unlikely, however, that under the experimental conditions atmospheric humidity immediately over the developing spores was ever far from saturation until the agar itself dried out and all plants perished. Hence it seems reasonable to suggest that the rare occurrence of bracken prothalli and sporelings in nature may to some extent be a consequence of the capacity of bracken spores to germinate immediately, when the young plants produced cannot endure subsequent desiccation.

During the first wet spell all spores shed on the ground will germinate immediately, only to perish the next time the surface dries out. Localities with sufficient light and a more humid atmosphere, such as rabbit burrows and ditches, may then represent the only kind of habitat where prothalli may survive their first critical growth period.

Sporophyte generation. Goebel (1900) expressed the view that the primary or juvenile leaves of ferns are strictly comparable in structure with adult forms but that their development must be regarded as arrested. Wardlaw (1945) also succeeded in inducing the production of 'juvenile' leaves by defoliation experiments. In the present experiments it was observed that the degree of differentiation of the primary leaf (i.e. the amount of subdivision of the pinna) depends to some extent on the size of the mother prothallus. A sporeling on a large prothallus shows much greater morphological differentiation of the primary leaf, which might even attain the form of the third leaf of a more nearly average sporeling, while that on the sporeling from a starved, nitrogen-deficient prothallus is correspondingly reduced (Pl. XVIII, A).

Effect of light on leaf expansion. The unfolding of the bracken fronds appears, at least in part, to be controlled directly by light. In the water-culture experiments leaves started to expand while still several inches below the surface and as a consequence became injected as soon as the stomata

developed. When the surface of the water was shaded leaf expansion proceeded only after emergence into the air and light.

Phototropism of the rhizome. The bracken rhizome shows a marked negative phototropic reaction. This was seen in water as well as sand culture. When shaded water cultures with almost horizontal rhizomes were exposed to light, all rhizome tips turned away from its source. In sand culture, rhizome tips reaching the side of the pot almost invariably grew vertically upwards. As soon as they reached the sand surface or shortly before doing so, they turned sideways into the sand again.

Dominance of older leaves. It is well known that normally bracken produces only one crop of leaves per annum. Removal of these leaves by cutting (Braid, 1935) induces younger leaf-buds to grow up, and if these are cut in their turn even a third crop of leaves may be produced. This observation has been confirmed here by defoliation of sporophytes in their second year of growth. The inhibitory effect of older bracken fronds on younger ones appears to be identical with that found by Goodwin (1937) and Ashby (1948) in flowering plants. In the first-year sporophyte the effect is much less marked and some young leaves are normally produced almost to the end of the season. However, this difference is only one of degree of inhibition, for Albaum (1938) has demonstrated that inhibitory effects are exerted even by the first sporeling leaf. He has also demonstrated the probability that the inhibition is due to auxin. 3-indole acetic acid in lanolin paste applied to the cut leaf-stump is as effective as the primary leaf itself in delaying the growth of the next leaf-primordium.

Sporophyll production. Sporophyll production in bracken can take place from the second year of growth onwards. It is unlikely that the size of the plant is directly related to the production of sporophylls, considering the large dimensions attained by some bracken sporelings in their first year. The suggestion would appear attractive that a dormancy period must intervene before reproductive organs are produced. It would be interesting to compare these phenomena with those in bracken growing under tropical conditions. Apart from this hypothetical dormancy requirement, evidence has been obtained that bracken will produce sporophylls only if given fairly high light intensities. As far as has been observed by the author, sporophylls do not occur in nature in shaded situations, and this has been confirmed experimentally by shading a few second-year plants while sprouting in the spring. Here only the controls in full light produced sporangia. Once sporangial development has begun, however, even complete darkness will not check it. Goebel (1900) also has hinted that high light intensities are required for sporophyll production.

Preliminary Experiments on the Mineral Nutrition of the Prothallial Generation

The following two experiments were undertaken to determine the effects of complete omission of the major nutrient elements from the culture

media on (1) germination of spores and (2) growth of the prothallial generation.

A. *Prothalli planted into sand cultures deficient in* N, P, K, S, Ca, *and* Mg

Prothalli grown from spores on mineral nutrient agar under the standard conditions described for the main experiment were transplanted into sterile crystallizing dishes each covered with a glass lid and containing sand saturated with the same standard nutrient solution employed before, but completely lacking in one each of the above elements. All other ions were kept constant, the only exceptions being chloride and sulphate.

Of each treatment three replicate dishes were grown.

At the time of transplanting the prothalli had a diameter of about 1 mm. No quantitative data were collected, but the plants were regularly inspected and symptoms noted.

Nitrogen deficiency. As early as 1 week after transplanting the prothalli appeared to be reduced in size compared with the control sets. Later the small plants became very yellow and their rate of growth was slow. However, after nearly 2 months they began to produce tiny sporelings having exceedingly minute primary leaves. The total area of the primary leaf did not usually exceed 1–2 $mm.^2$ Some 3–4 weeks after emergence of the sporeling the prothalli became very pale and died. The minute sporelings, however, persisted and some produced a second leaf, equally minute. The primary root of these sporelings was remarkably long.

Phosphorus deficiency. The first symptoms of lack of phosphorus did not appear until the third week after transplanting, but the effects became very severe a short time later. The prothalli became very pale greyish-green and almost transparent. Shortly afterwards growth ceased altogether, the diameter attained by the largest plants never exceeding 5 mm. Some antheridia had been formed, but none of the plants produced sporelings.

Potassium deficiency. Lack of this element led to a reduction in size of the prothalli, and especially of the young sporelings. Fewer sporelings were produced than in the control dishes, and some prothalli died prematurely, but the effects were comparatively slight compared with those of N and P deficiencies.

Sulphur deficiency. No sulphur deficiency symptoms were demonstrable in the prothallial generation, but the young sporelings produced showed them at an early stage. They became stunted and the primary and later leaves were markedly chlorotic.

Magnesium deficiency. Omission of Mg from the culture solution had only a slight effect on both prothalli and young sporelings; it caused a moderate degree of chlorosis, but other symptoms were not clearly defined.

No calcium deficiency symptoms were observed.

B. *Sowing of spores on standard mineral nutrient agar deficient in* N, P, K, Mg, Ca, S, Fe, B, Mn, *and* Zn

Washed spores were sown under sterile conditions by means of an atomizer on agar washed with distilled water and contained in Petri dishes. A circular

disc of agar $1\frac{1}{2}$ in. in diameter was removed from the dishes and the cavity was filled and regularly replenished with sterile distilled water so as to keep the agar fully saturated.

Each of the solutions incorporated in the agar was completely deficient in one of the elements under consideration; all unwanted variability of ions was borne by the chloride and sulphate radicals.

Each treatment was replicated four times.

No quantitative data were collected, but observations were made at regular intervals.

Results

Within 10 days from sowing, germination was general in all cultures, but possibly was delayed very slightly in the potassium deficient cultures.

Nitrogen deficiency. Lack of nitrogen was mainly effective in reducing the size of plants at all stages of growth. Thus the average number of cells in the prothalli after 6 weeks of growth amounted to only about 70 at a time when the controls had reached a diameter of 1–2 mm. Four months after sowing, the prothalli had reached a diameter of about 3 mm. and several were producing sporelings with small awl-shaped leaves. The colour of the prothalli was yellowish, but the number of chloroplasts did not seem to be reduced.

Phosphorus deficiency. Deficiency symptoms of phosphorus were much more severe than of any other element. Growth rate was extremely reduced and few prothalli produced more than 50 cells during the whole period of over 4 months. Many of them failed to form plates at all; some formed irregular 3-dimensional conglomerations of cells, while a few never produced more than 3 or 4 cells. Rhizoids of these plants were unusually long, some exceeding 20 times the diameter of the prothallus. Reduction in the amount of chlorophyll was a striking feature, and (unlike the yellowing under nitrogen deficiency) this appeared to be due to reduced numbers of chloroplasts per cell. At a later stage chlorophyll was found only in the youngest portions of the plant. Finally, or by the time the experiment was concluded, the small meristematic cells normally present near the apex of the prothallus had become fully enlarged and growth had ceased altogether. None of the plants produced a sporeling and no antheridia or archegonia were found on any of them.

Potassium deficiency. The main effect noted in the absence of this element was again reduction in size together with some slight chlorosis. After some time irregularities in growth appeared and eventually there was a tendency for the apical region of the prothallus to grow out into a cylindrical structure. Proliferation from cells in the older parts of the prothallus was frequent, as well as death of the older parts. Although many antheridia were formed, no sporelings were produced.

Sulphur deficiency. Deficiency symptoms of sulphur consisted in slight chlorosis and moderate reduction in size. Antheridia and archegonia were formed normally, and some sporelings were produced.

Iron deficiency. Lack of iron caused some chlorotic symptoms, but beyond this no other effects were noted. Sporelings were eventually present.

Deficiencies of the other elements omitted from the nutrient solutions did not lead to any clear symptoms. It is possible that the repeated washing of the agar with distilled water was insufficient to remove all the traces of salts required by the prothalli for normal growth.

In the later stages of the experiment the dishes became heavily contaminated with algae and fungi, and it had eventually to be abandoned.

Conclusions

The only comparable experiments carried out on the nutrition of fern prothallia are those of Nagai (1914), Czaja (1921), and Prantl (1881). Several species of ferns were used by these authors, but only Czaja used *Pteridium aquilinum* in one experiment. He tested prothallia on N-deficient media, but his recorded observations are confined to the presence or absence of archegonia and antheridia. Transfer to N-deficiency conditions usually caused a cessation of archegonial production and antheridia appeared. Both Nagai and Prantl also noted a reduction in size of prothallus when cultures lacked nitrogen; at the same time they found much-increased starch contents, while the production of archegonia was entirely suppressed. Under those conditions organized apical meristems were altogether absent, but could be reestablished by transfer to media containing available nitrogen. Nagai noted less intense effects on production of sex organs when phosphorus was deficient, but starch contents were low compared with the controls. Prantl even found increased starch contents when N and P were absent or deficient simultaneously. From most of these data it appears that the severity of deficiency conditions produced by these authors was considerably less than that in the agar cultures described above.

From the present data it is evident that phosphorus supply is of great importance to the prothallial generation; it will be shown later to be equally important to the sporophyte. Substantial supplies of nitrogen and potassium are similarly essential, but under the experimental conditions phosphorus supply was the dominant factor. Nitrogen effects on reproduction do not seem to be as severe in bracken as those on other ferns noted by the authors cited above.

Mineral Nutrition of the Sporophyte Generation

Main Experiment of the 1946 *Season*

Design of experiment

The main experiment of the 1946 season consisted of a factorial combination of three variants: 3 levels of potassium × 2 levels of phosphorus × 3 solution types, comprising 18 treatments in all.

Potassium and phosphorus were chosen for the following reasons. It has sometimes been claimed that bracken has a high potassium requirement—an

inference drawn from the fact that a high potassium content is frequently found in the ash from portions of the plant (Wetzel, 1938). Preliminary experiments in 1945 revealed large effects of phosphorus deficiency on the growth of the bracken sporophyte and suggested that it might be an important factor determining the growth of bracken in the field. The three solution types were used because it has been shown by Richards and Shih (1940) that effects of mineral deficiencies, especially those of potassium, differ considerably with the composition of the basal solution employed. The three types chosen were widely different in composition: they were designated (*A*), (*C*), and (*M*) in accordance with the terminology employed previously at this Institute. In the first type, *A*, the main anions were supplied as sodium salts, calcium being maintained at a low level. In the second type, *C*, calcium salts were given in the absence of sodium, while in the third type, *M*, nitrogen and phosphorus were supplied as ammonium salts in the absence of sodium and the presence of the same quantity of calcium as in type *A*. The choice of these three solutions enables also some inferences to be drawn regarding the effect of pH on the growth of bracken, a factor sometimes stated to be of considerable importance to this fern. The different rates of anion and cation absorption in these solutions resulted after a short time in widely divergent pH values of the cultures.

The actual nutrient combinations used are presented in Table II together with the nomenclature employed. The solutions were chosen to be identical with those employed by Richards and Shih (1940) for barley and Richards (1942) for flax so that comparisons made between the effects on bracken and those on flax and barley might be as close as possible. The only exception is in the lower level of the phosphorus which was only one-half of that of the 1940 experiment. The full dosages of the main nutrients were found to be adequate for barley and flax and proved to be so for bracken also in preliminary experiments during the 1945 season. However, about half-way through this experiment the fully fertilized plants of the *A* series began to show symptoms not unlike those of potassium deficiency, and in order to test the possibility of a still higher demand for potassium, on August 19 two plants from each of the high nutritional series in all three solutions were given sufficient potassium to bring their supply up to three times that of the standard dose; however, no benefit resulted from this extra amount of *K* and the plants did not differ from the controls at standard level in each solution. The three levels of potassium employed were: $K1$ = the standard dose, $K3$ = 1/9 standard dose, and $K5$ = 1/81 standard dose. Of the two levels of phosphorus the standard is designated (*H*), the lower level (*L*) is 1/10th the standard dose. Nitrogen and magnesium were kept constant throughout, while sodium and calcium levels in the various low phosphorus series were made up to the corresponding levels in the high phosphorus series by the use of sulphate and chloride respectively. Hence sulphate and chloride were the only variable ions besides those under investigation. The initial pH of solutions *A* and *M* was adjusted to 5·5 approximately by supplying 5 per

cent. of the phosphorus as the monohydrogen phosphate and the remainder as the corresponding di-hydrogen salt. The initial pH of solution *C*—which was not adjusted—was found to be lower, lying between 4·0 and 4·5. The solutions were applied to the pots in three equal doses on the following dates: June 3–6, July 2–3, July 29–30, i.e. the first dose was given just prior to planting out.

TABLE II

Nutritional Scheme. Salts in g. per pot

Salts.	Solution *A*		Solution *C*		Solution *M*	
	HA	*LA*	*HC*	*LC*	*HM*	*LM*
$NaNO_3$	9·11	9·11	—	—	—	—
$NaH_2PO_4.H_2O$	1·098	0·110	—	—	—	—
$Na_2HPO_4.12H_2O$	0·15	0·015	—	—	—	—
$Na_2SO_4.10H_2O$	—	1·274	—	—	—	—
$Ca(NO_3)_2$	—	—	8·79	8·79	—	—
$CaH_4(PO_4)_2.H_2O$	—	—	1·06	0·106	—	—
$CaCl_2.6H_2O$	0·37	0·37	0·37	1·199	0·37	0·37
$MgSO_4.7H_2O$	1·25	1·25	1·25	1·25	1·25	1·25
NH_4NO_3	—	—	—	—	3·928	4·246
$(NH_4)H_2PO_4$	—	—	—	—	0·918	0·092
$(NH_4)_2HPO_4$	—	—	—	—	0·055	0·0055

Potassium levels, g. per pot.

$K1 = 1·85$
$K3 = 0·206$
$K5 = 0·023$

Minor elements per pot. *All pots*

H_3BO_3	0·0286
$MnSO_4.4H_2O$	0·0204

Nomenclature employed

	Sodium solution		Calcium solution		Ammonium solution	
	High phos.	Low phos.	High phos.	Low phos.	High phos.	Low phos.
High potassium	*HAK*1	*LAK*1	*HCK*1	*LCK*1	*HMK*1	*LMK*1
Medium potassium	*HAK*3	*LAK*3	*HCK*3	*LCK*3	*HMK*3	*LMK*3
Low potassium	*HAK*5	*LAK*5	*HCK*5	*LCK*5	*HMK*5	*LMK*5

Reduction of error

At the beginning of the experiment, when the young sporelings were transplanted into the large glazed earthenware pots, there were still noticeable differences in size between the individual plants in spite of the earlier rigorous selection for uniformity.

In order further to reduce the experimental error due to differences in initial size, the plants were divided into three size classes which were later treated as blocks in the statistical analyses. Spare plants were also grown in each treatment to replace accidental losses of replicates.

Collection of data

Five samples were taken during the growing season in addition to a preliminary one (Table III) recording the initial characteristics at the time of

planting out. Later three replicate pots per treatment—one from each block—were sampled at four times during the growing season. These samples were carried out at fortnightly intervals on the following dates: July 23–4, August 6–7, August 20–1, September 3–5. A final harvest of a further six replicates per treatment (i.e. two from each block) was carried out on October 16–22.

TABLE III

Preliminary Sample (June 9–11, 1946). Three replicates per block

Number of green leaves	5·7
Number of dead leaves	0·3
Number of unexpanded leaves	1·1
Total leaf number	7·1
Number of leaf-buds	3·3
Number of rhizome branches	2·4
Total leaf area, sq. cm.	20·5
Total dry weight, g.	0·119
Leaf fraction, % total dry weight	60
Rhizome fraction, % total dry weight	11
Root fraction, % total dry weight	29
Leaf water content, % dry weight	477
Rhizome water content, % dry weight	730

At each sample were recorded the numbers of green leaves, dead leaves, leaf-buds, and rhizome branches longer than 0·5 cm. Fresh and dry weight of these parts, as well as the dry weights of the roots, were also noted. In addition to these data an estimate of total leaf area per plant was made from photographs taken at the sampling times; details of this method are given below. Besides these samples, counts were made at weekly intervals throughout the experiment of the number of living, dead, and newly expanded leaves on 6–7 plants from each treatment.

The pH of the solutions in the various cultures was determined by glass electrode withdrawing small quantities of liquid from each pot. These observations were made on the occasions of the first two samples and the final harvest.

Finally, the rhizome material of the third sample and the final harvest were analysed for their starch content by Hanes' method (1936).

Measurement of leaf area

It is a matter of some difficulty to determine with any degree of accuracy the area of a leaf of such complex shape as that of bracken. A planimeter was used for measuring the area of the few small leaves present at the time of the preliminary sample, but the use of this instrument is clearly out of the question for an average-sized bracken leaf. A method of reproducing the leaves on sensitized paper followed by cutting out and weighing of the paper is equally unsuitable. Direct estimation by means of a photo-electric cell as used by Kramer (1937) was likely to be inaccurate, since many of the leaves are relatively transparent and the amounts of light transmitted would differ

with age and with treatment. It is unlikely that the use of colour filters would have corrected sufficiently the errors due to transparency, and in any event a direct method such as this would have yielded only a single estimate of the area of each plant and no permanent record would have been obtained. Permanent records eventually proved to be invaluable for an accurate determination since the estimation could be repeated several times. The chief difficulty in all photometric estimations is that of an even and reproducible illumination. For this reason the use of artificial light is imperative, but even so fluctuations of the mains voltage were found to be so great that single readings would have been valueless.

The method finally adopted consisted in taking photographs of the leaves at the sampling times, and subsequently estimating their areas photometrically. Fig. 1 shows the arrangement for photographing the leaves. The leaves were spread out evenly between two glass plates supported horizontally. Illumination was provided by light reflected upwards through the glass from a sheet of white paper itself illuminated as uniformly as possible by four lamps. The leaves were then photographed from above on process plates (6·0 cm. × 4·5 cm.) to secure maximum contrast. After development these plates were partially bleached to remove any silver deposited inside the leaf image due to transparency, and subsequently intensified, the final negatives showing the leaves as perfectly transparent areas in a completely opaque background (see Pl. XVIII, B).

The arrangement for the photo-electric estimation of the areas from these plates is shown in Fig. 2. Light from a 200 W. pearl lamp placed in a head-on position was diffused by a screen of flashed opal glass covering a rectangular aperture in a light-tight partition wall. The distal side of this aperture was just covered by the plate whose transmission was to be estimated. Light passing through this plate fell on the sensitive surface of a VA 39 Cintel photo-electric cell. As the light passing through the plate was quite diffuse there was no need to use condensers, &c., to concentrate the light on the cell, the energy received being directly proportional to the total transmitted. The current produced was read directly from a micro-ammeter.

The cell was standardized directly against known areas and its linear response confirmed over the range covered (24 times the minimum value). Using a small standard transmission area the apparatus was tested for uniformity of light intensity over the whole rectangle covered by the plate. During the preliminary trials it was found that variations in the mains voltage of up to 20 per cent. caused changes in light intensity of well over 50 per cent. (of the upper values) for the same transmission area. To overcome this difficulty the individual plate readings were alternated with those for a standard area and their ratio determined. All these readings were duplicated and the mean value calculated. Use of a constant voltage would render this method very much more satisfactory while reducing the time for each measurement to one quarter. The actual errors of estimation did not, however, exceed 3–4 per cent.

The scale of reduction on the photographic plate was measured from pieces of card of known length photographed with the leaves at the sampling times.

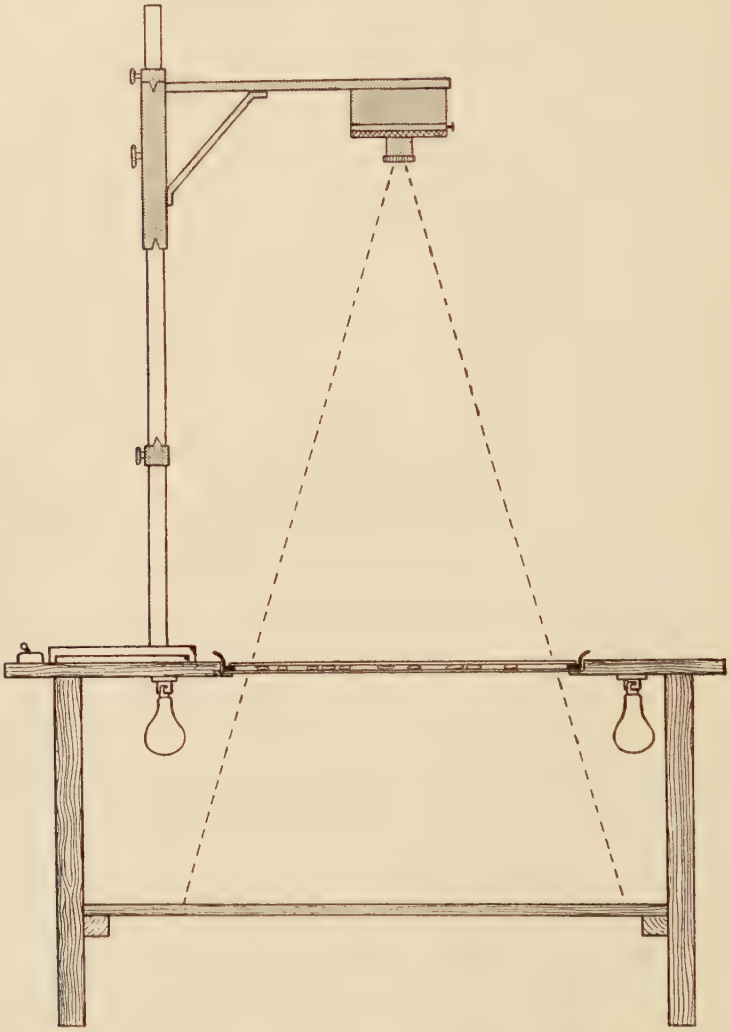

FIG. 1. Arrangement for taking leaf-area photographs.

Two reduction factors were used, depending on the size of the leaf sample: $\times 11{\cdot}5$ and $\times 5{\cdot}9$. In all cases labels indicating treatment and replicate number were photographed with the plates (cf. Pl. XVIII).

Effect of weather conditions

It should be pointed out here that all the open-air pot cultures were severely affected by the unusual climatic conditions prevailing during the

summer of 1946. Usually in pot culture experiments the moisture content of the pots needs to be maintained by supplying tap-water. During the 1946 season, however, gain of water through rainfall was consistently in excess of that lost by evaporation, and an ever-increasing surplus had to be drained in order to prevent the pots from becoming waterlogged. The solutions thus removed were stored in bottles and returned again to the pots when occasion demanded. At one stage over 4 litres of solution per pot had been thus collected. Since the culture solution remaining in the pot itself was considerably diluted, the contents of the bottles were percolated through the sand twice a week, in order to avoid the possibility of lack of nutrients other than those intended.

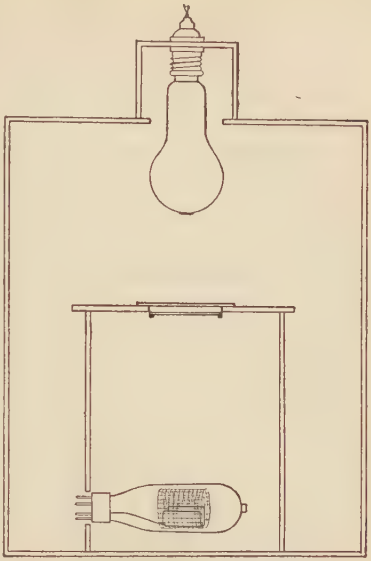

FIG. 2. Arrangement for photo-electric estimation of leaf areas from photographic plates.

EXPERIMENTAL RESULTS

General observations

Day-to-day observations on the general appearance of plants in different nutrient treatments were confined to the leaves, i.e. the only parts visible above sand level. The appearance of rhizomes and roots could, however, be compared at the sampling times, and the lack of frequent observations of the underground portions proved to be of very little importance, as one of the most striking effects was the uniformly healthy appearance of these parts, and especially of the rhizomes, at all treatment levels and in all three solutions. Even in treatments where the leaves were dying rapidly the rhizome itself died only after all the tops had perished some time previously. This might perhaps be regarded as an indication of the relative powers of competition for nutrients between leaves and rhizome.

The first symptoms of treatment differences appeared after about 3 weeks of differential conditions, slight reductions in size due to phosphorus deficiency being the first effects to be noticed. Very soon afterwards, potassium deficiency and solution-type effects became obvious also.

1. *Phosphorus deficiency.* For the first few weeks, diminished size of individual leaves and of the whole plant were the only signs of deficiency. Later the small leaves tended to become quite hard and rather brittle, a feature especially notable in the *A* solution. At first their colour was a slightly lighter hue of green than that of the controls, but this was soon reversed and for the rest of the experimental period the plants were conspicuous by their

dark green foliage. After 6 weeks' growth under deficiency conditions the older leaves exhibited brown speckles on laminae and petioles as well as increased hairiness; with advancing age of the leaf these spots tended to increase in size and larger necrotic areas were formed. In the *C* solution only the upper surface of the leaf appeared to be thus affected. The dead leaves were of an unusually dark brown, in some cases almost black. Generally, the foliage appeared to be densely clustered and the leaves arose only from the centre of the pot. The number of green leaves present was small, but their length of life was not especially shortened, the total number produced during the season being much reduced. These effects were found in all three solutions, but the size reduction compared with the corresponding high phosphorus series was more marked in the *M* solution than in the other two.

In the LK_5 treatments, especially the *A* and *M* solutions, mixed symptoms of potassium and phosphorus deficiencies appeared; later, the effects of potassium shortage predominated in the *M* and especially the *A* solution, but in the *C* solution potassium-deficiency symptoms remained subsidiary to those of lack of phosphorus.

2. *Potassium deficiency.* The most obvious symptom of lack of potassium at the K_3 and K_5 levels was an accelerated death-rate of the older leaves, which led in time to the complete denudation of the old centres of the plants, which were surrounded by a fringe of green leaves. The effects which differed very markedly with the different solution types were most pronounced in the high sodium solution (*A*). Here the older portions of the mature leaves turned a yellowish colour, beginning at the petiole and spreading towards the tip. The yellowing was quickly followed by a dark purplish-brown discoloration and eventually by the death of the leaf at sand level. It frequently happened that the tip of the leaf was still green and turgid when the base was already completely decayed and parting from the plant. The new leaves produced had generally a rather curly, compressed, and succulent appearance. At the lowest potassium level (K_5) one-third of the plants died altogether in the high P solution and one-sixth at the lower P level. Towards the end of August the symptoms of potassium deficiency in the HAK_3 and HAK_5 treatments had reached their maximum severity and after this time some recovery set in, which was largely maintained for the rest of the experiment. At the lower phosphorus level (LAK_5) the plants reached the maximum deficiency symptoms rather more slowly than did the HAK_5 plants, but the final appearance differed little in the two series.

In the *M* solution the plants were considerably larger. Early death of leaves was equally marked, but curiously enough the sequence of events leading to it was the exact reverse of that in the *A* solution. The tips and margins of the leaf were affected first, the petiole remaining alive to the last, i.e. the meristematic portions of the leaf were first affected by lack of potassium in the ammonium solution.

Symptoms in the calcium solution were at first rather like those of the

ammonium type, but later resembled those of the sodium solutions more. They were less severe than in the other types.

For all three solutions the differences between the two lowest potassium levels were in degree only and not of kind.

3. *Solution types.* The sole difference in visual effects between the three solutions covering the whole range of P and K levels was a markedly light colour of the dead leaves and underground portions of plants grown in the *M* solution.

Other effects were confined to those plants grown at high nutrient levels, i.e. the *HK*1 series.

The foliage of plants grown on the *M* solution was the largest, and it was generally somewhat darker green than in the other two; the individual leaves also had a rather more delicate appearance. In the *A* solution plants were rather smaller and leaves tended to be more succulent and curly; they often showed some slight chlorotic symptoms; and death of leaves occurred earlier than in the other two types—effects not altogether unlike those associated with potassium deficiency. In the high calcium series leaves appeared to be normal in colour and shape; on the whole they did not differ much from the *M*-type plants.

4. *Changes due to age.* As the rhizomes increased in length the new leaves, especially those of the high manurial series, tended to be produced farther from the old centres of the plant and closer to the edge of the pot. Generally the size of the leaves increased with the advancing age, but this effect was much less marked in the deficient series. Towards the end of the season leaf production rapidly fell to a low level, but even by the end of October, when the experiment was ended, it had not ceased altogether. This observation contrasts markedly with others made on plants in their second year of growth, when leaf production generally ceases as soon as the first crop has expanded.

Leaf counts

Every week the total number of living leaves present was counted on each of six or seven replicate plants from every treatment; in addition the number of new leaves was determined, i.e. leaves which had unfolded their lowest pair of pinnae and were beginning to contribute to the carbohydrate accumulation of the plant. The total number of leaves produced by any one plant was obtained by adding the number of new leaves to the previous week's total. The number of dead leaves was calculated as the difference between the overall total and the number of living leaves extant. Determination by difference was necessary since the dead leaves gradually decayed and disappeared altogether after some time.

The results of these counts are presented in graphs (Fig. 3, *a–r*), the upper curve representing the mean total number of leaves produced per plant, the lower curve the total number of dead leaves. The slopes of these curves indicate the rates of production or death of leaves; the vertical distances between the two members of each pair of graphs measure the number of

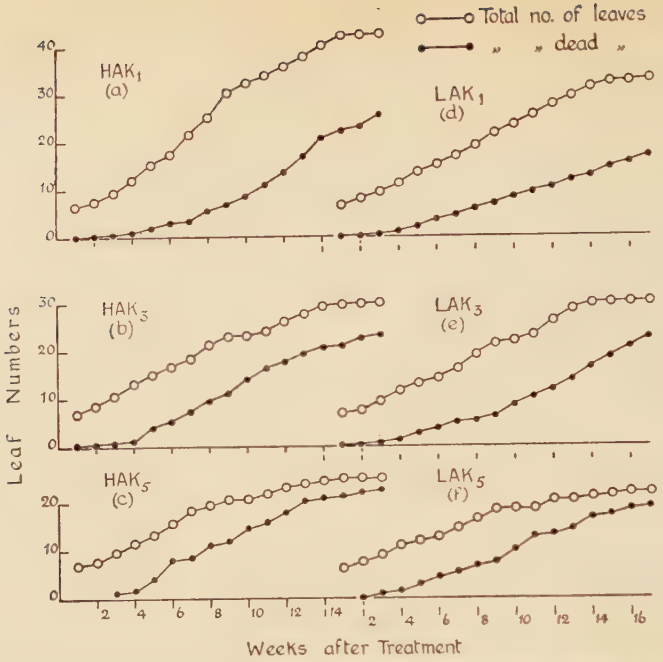

FIG. 3, *a–f*, numbers of living and dead leaves in the *A* series (means per plant).

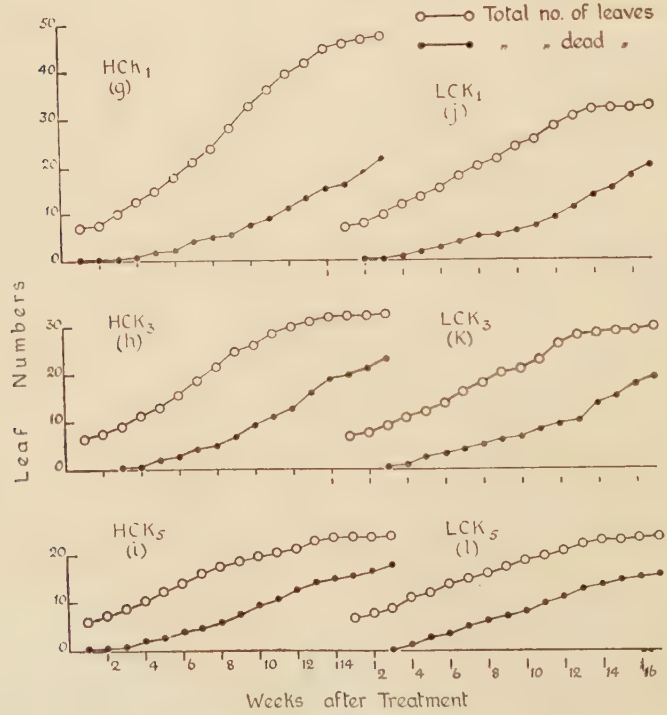

FIG. 3, *g–l*, numbers of living and dead leaves in the *C* series (means per plant).

living leaves present at any time, and the horizontal distances give an estimate of the length of life of individual leaves. The means of these longevity values have been calculated for each treatment and will be discussed below (Table IV).

The total number of leaves produced per plant in the three high manurial series is large, the maximum number being produced in the *M* solution (60

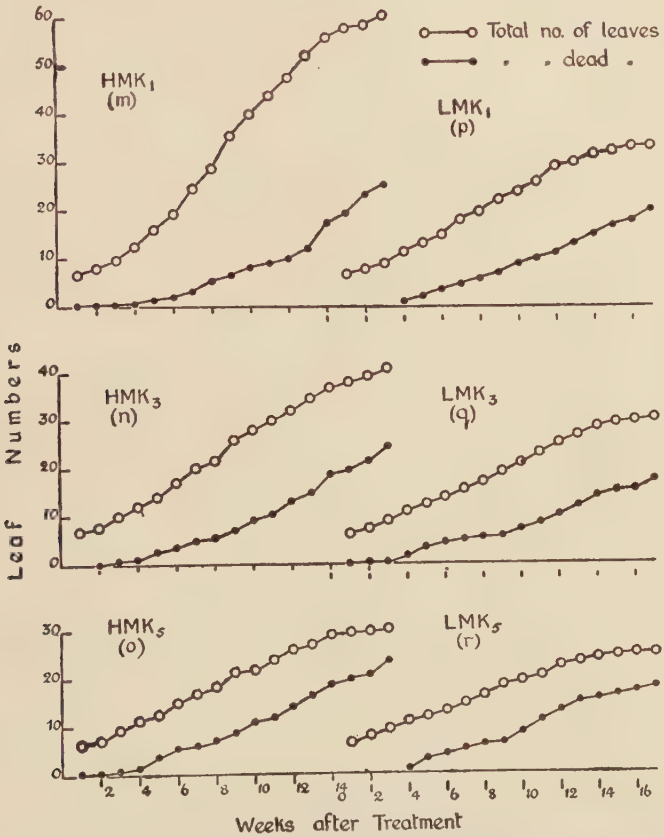

FIG. 3. *m–r*, numbers of living and dead leaves in the *M* series (means per plant).

leaves at the end of the season); in the *C* and *A* solutions the totals are lower (47 and 42) but still considerable. Of course, these numbers do not represent large fronds only but also include smaller fronds produced later in the season at the old centres of the plants. Of these final totals 42 per cent. were dead in the *C* and *M* solutions and 62 per cent. in the *A* solutions.

1. *Phosphorus deficiency.* Lack of phosphorus causes a very marked depression of the leaf-production rate. At the *K*1 level the reduction is of the order of 25 to almost 50 per cent., and though the effect is much weakened at the low potassium levels it is still noticeable. The reduction is especially marked in the ammonium solution (*HMK*1–*LMK*1) and least in the sodium solution

(*HAK*1–*LAK*1). The relative death-rate is only affected slightly by a lowering of the phosphorus supply. Even though it is only a little below the rate of leaf production, the number of living leaves per plant rises slowly until a few weeks from the end of the experiment when the rate of dying overtakes that of production. The time of this acceleration of the leaf death-rate in the *L* series depends on the potassium level and solution type; it is especially early in the *LAK*5 plants. Generally the maximum number of living leaves is reached at about the same time as in the fully manured series, but later than in the corresponding potassium deficiency levels with a high phosphorus supply. This is also seen in the larger number of leaves surviving until the final harvest in the *LK*5 as compared with the *HK*5 treatment.

2. *Potassium deficiency.* The absolute rate of leaf production is equally reduced by a diminished potassium supply. At the high phosphorus level the number is lowered by one-third at the *K*3 level and by half at the *K*5 level. In the low phosphorus series a marked decrease (again about one-third) is also found at the lowest potassium level (*K*5) but not at the *K*3 level. The increase of the relative death-rate mentioned previously is even more obvious from the graphs. Thus the number of leaves dying every week is equal to or higher than that in the fully manured series with their much greater rates of leaf production. This effect depends largely on solution type and is especially marked in the *A* and *M* solutions, much less so in the *C* solution. The serious result of this acceleration of the relative death-rate becomes very striking by the end of the season when the number of surviving leaves falls to a very low figure. Although a much higher proportion of leaves survived in the *HK*1 treatments, the absolute numbers of dead leaves at this time were closely similar in all high phosphorus series. The effects among the potassium treatments at the lower phosphorus levels are somewhat similar, but all the results are overshadowed by the common factor of *P* deficiency.

3. *Solution types.* The type of solution also has a marked effect on leaf-production rates. Plants in the *M* solution at the high nutritional level carry the highest number of leaves, and this effect is maintained relative to the other two solutions at all investigated nutrient levels, though it is largely eliminated by conditions of phosphorus shortage. The *C* and *A* solutions are more nearly equal as regards leaf-production rate, but death-rate is usually appreciably lower in treatments of the *C* solution.

Longevity of leaves

The average length of life per leaf was calculated from the leaf-number graphs in the manner outlined above. This method rests on the assumption that the leaves die in the order in which they are produced. Of course, this is not strictly true in every case, but observations have shown that it is sufficiently reliable for a calculation of the average longevity of a large number of leaves. Furthermore, this estimate includes only those leaves which have actually died by the end of the experiment, and especially in the case of the high manurial treatments this may have led to a slight underestimate of

longevity, for it appears from the slope of these curves that longevity increased somewhat with age of plant. The result expressed as weeks after expansion of the first pair of pinnae are shown in Table IV.

TABLE IV

Longevity in Weeks

	H				*L*			
	*K*1.	*K*3.	*K*5.	Mean.	*K*1.	*K*3.	*K*5.	Mean.
A . .	7·5	5·8	4·6	6·0	8·9	7·3	6·2	7·5
C . .	9·1	7·2	7·4	7·9	9·3	8·6	8·0	8·6
M . .	8·9	7·7	6·4	7·7	8·1	8·6	7·2	8·0
Mean . .	8·5	6·9	6·1		8·8	8·2	7·1	

They may be summarized as follows. The mean effect of high dosage of potassium at both levels of phosphorus and in all three solutions is to increase longevity by almost 30 per cent. over the $K5$ and about 15 per cent. over the $K3$ levels. In the A solution this effect is greatest, the percentages being about 52 and 24; it is least in the C solution (25 per cent. and less than 4 per cent.). At the lower phosphorus level length of life is appreciably affected by potassium dosage only at the lowest level.

In the presence of sufficient K, changes of phosphorus nutrition have little effect over the range investigated, but combined with lack of potassium increasing doses of P reduce the length of life. Hence the mean effect of phosphorus seems to be a slight decrease of longevity.

Again, solution type is an important factor determining longevity. It increases in the following order of solutions: $A < M < C$. This order is maintained over all levels of P and K with the exception of the $HK3$ and $LK1$ treatments where the orders are $A < C < M$ and $M < A < C$.

Numbers of leaf-buds and unexpanded leaves and numbers of rhizome branches longer than 0·5 cm.

The numbers of unexpanded leaves and leaf-buds on the rhizome and numbers of rhizome branches were counted at sampling times. These counts were made in order to obtain a measure of the numbers of meristematic centres in the plants and thus of meristematic activity generally. Bower (1923–8) mentions that leaf-bud production in bracken is generally associated with branching of the rhizome. But this applies only to the main rhizome branches; in the old centres of the plants, leaf-buds are produced without any branching. Hence numbers of rhizome branches tend to be considerably fewer than the total number of leaf-buds and leaves. In any case, as it is somewhat difficult to be certain of the first stages of rhizome branching, only laterals longer than 0·5 cm. were counted.

The numbers of leaf-buds, not being subject to an arbitrary size limit, have been analysed statistically using the $\log(x+1)$ transformation, and with one exception the treatment effects presented below have been found

significant when compared with experimental error and all significant higher-order interactions involving the factor or factors concerned.

The age effect is highly significant, bud numbers increasing throughout the season. When the mean logs of the numbers are plotted against time (Table V, Fig. 4, *a*) it is seen that the rate of increase falls apparently regularly until the fourth sample; but by the time of the final harvest the rate has very much increased again. This effect seems to be quite unrelated to the general size increase of the plants as is seen from a comparison with the log total dry-weight graph (shown also), which shows no sudden change in the rate of increase. Also, comparison of the graphs of the antilog of the mean leaf-bud numbers and mean numbers of rhizome branches indicates that the effect is mainly confined to the former (Fig. 4, *d*). However, from a comparison with the leaf-number graphs above (Fig. 3) it becomes clear that there is a strong negative correlation between the rate of expansion of leaves and bud numbers. It appears therefore that, when leaf expansion ceases at the end of the season, buds are still being differentiated at approximately the normal rate, and the autumnal decline in leaf production primarily affects the process of leaf expansion.

The interaction of age and K level (Table V (*d*) and Fig. 4, *c*) is also significant. Until the third sample, bud numbers increase at all three K levels, the rate depending on the actual dosage, but at S_4 there is a drop in numbers of the K_5 series. Finally, all three levels show the above-mentioned increase in the rate of production to the final harvest.

The highly significant P×K interaction (Table V (*b*)) and interaction diagram (Fig. 4, *b*) (see Richards, 1941) indicates a practically linear response of bud numbers to potassium supply, when phosphorus is in excess. With phosphorus deficiency numbers increase rapidly from the K_5 to the K_3 level, but further increase of K dosage has practically no effect. Actually numbers in the HK_5 series are below those of the LK_5, although the difference does not reach significance level. The ratios H/L of the untransformed values are for the three K levels:

	K_1	K_3	K_5
H/L . .	2·6	1·3	0·94

The K×Soln. interaction (Table V (*c*)) is also interesting. The response to K dosage is almost linear in the *C* and *M* series, but not so in the *A* solution, where the K_3 treatment lies much above the geometric mean of the two extremes. The ratios of the K levels give the following values:

	A	*C*	*M*
Linear term K_1/K_5	3·1	1·8	2·5
Curvilinear term $K_1 \times K_5/K_3^2$ . .	0·57	1·01	1·12

The actual ranges also are interesting, being greatest in the *A* and least in the *C* solution. The mean leaf-bud numbers in the AK_1 series are much smaller than in the MK_1 series, which is particularly high. The intermediate CK_1 treatment does not differ significantly from either extreme.

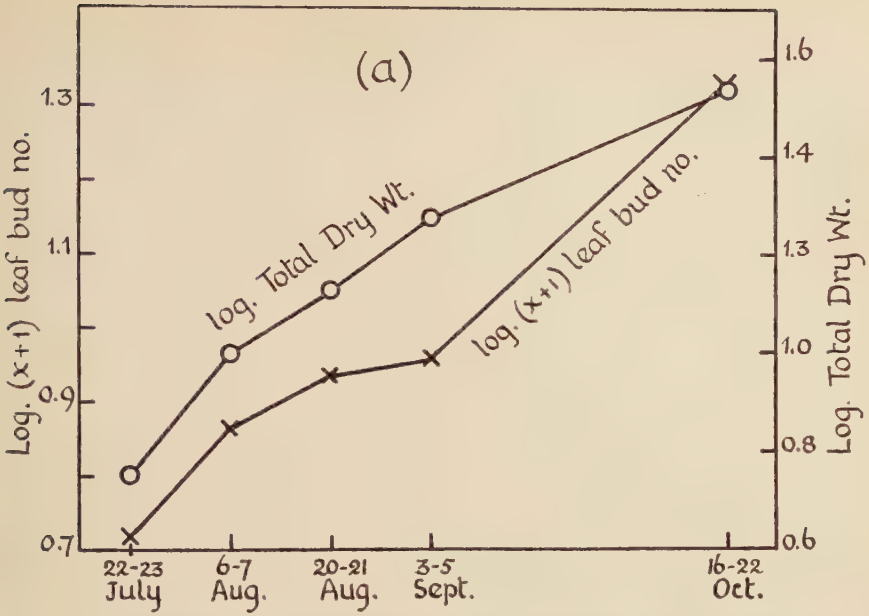

FIG. 4, *a*. Comparison of mean log($x+1$) leaf-bud numbers per plant with log total dry weight (decigrams) per plant.

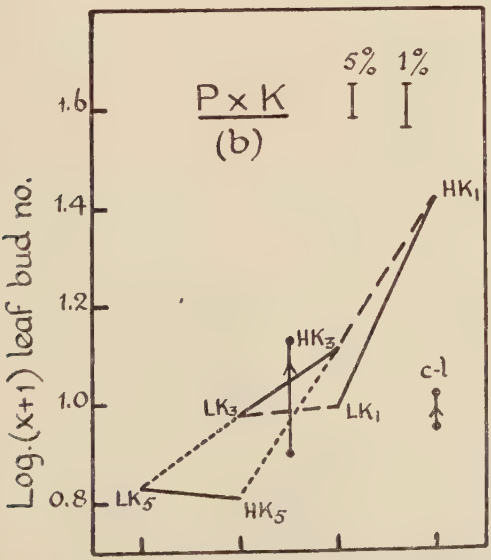

FIG. 4, *b*. Interaction between the effects of phosphorus and potassium supply on leaf-bud numbers (log($x+1$)), the length of the central vector indicates the magnitude of the linear term of the interaction, the second vector labelled $c-1$, the magnitude of the curvilinear term; both are positive.

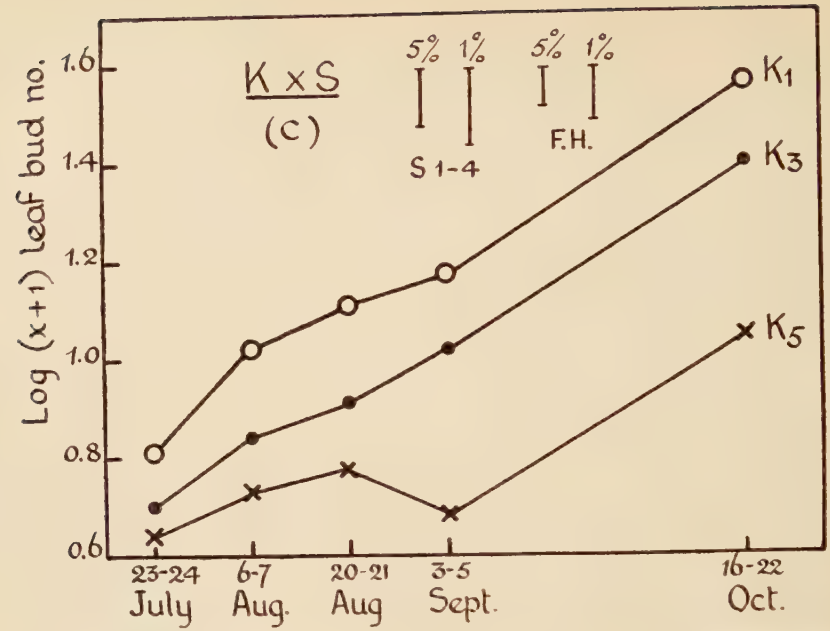

FIG. 4, *c*. Effect of potassium level on leaf-bud numbers ($\log(x+1)$) at different ages of the plant.

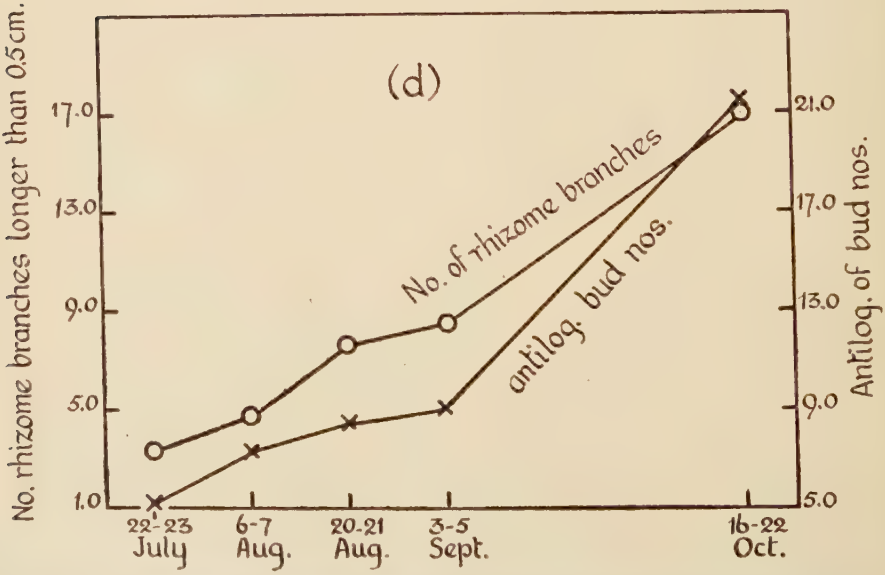

FIG. 4, *d*. Comparison of mean antilog of leaf-bud numbers per plant with mean numbers of rhizome branches (longer than 0·5 cm.) per plant.

Finally the third-order interaction P × K × Soln × Age reaches the 1 per cent. significance level. A description of this interaction will not be attempted here, for the simpler interactions already described are considerably larger than the more complex one, and the oversimplification involved by neglecting the latter is not serious.

All the effects described for leaf-bud numbers, with the single exception of the particularly large increase at the final harvest, are closely repeated in the rhizome branch numbers, which therefore require no further comment. They are shown in Tables VI (*a–d*).

Table V

Number of Leaf-buds and Unexpanded Leaves, $\log(x+1)$

$S1$–4 = sample numbers, F.H. = final harvest; other abbreviations are given in Table II. Significant difference values appropriate to the particular parts of the table are entered in section (*e*).

(*a*) *Age*: $S1 = 0{\cdot}716$, $S2 = 0{\cdot}863$, $S3 = 0{\cdot}930$, $S4 = 0{\cdot}960$, F.H. = 1·333
(Sig. diff. for S (2), for F.H. (1))

(*b*) $P \times K$
(Sig. diff. (2))

	H	*L*
$K1$ . .	1·414	0·993
$K3$ . .	1·108	0·979
$K5$ . .	0·807	0·833

(*c*) $K \times$ *Soln.*
(Sig. diff. (3))

	$K1$	$K3$	$K5$
A . .	1·135	1·008	0·639
C . .	1·204	1·075	0·949
M . .	1·271	1·048	0·873

(*d*) $K \times S$
(Sig. diff. for S (4), for F.H. (3))

	$S1$	$S2$	$S3$	$S4$	F.H.
$K1$ . .	0·806	1·016	1·106	1·172	1·560
$K3$ . .	0·700	0·843	0·913	1·022	1·392
$K5$ . .	0·643	0·730	0·770	0·685	1·047

(*e*) *Significant differences*

$P =$	*0·05*	*0·01*	*0·001*
(1) . .	0·048	0·063	0·081
(2) . .	0·068	0·089	0·114
(3) . .	0·083	0·109	0·140
(4) . .	0·118	0·155	0·197

TABLE VI

Mean Number of Rhizome Branches

(*a*) $S1 = 3{\cdot}3$, $S2 = 4{\cdot}7$, $S3 = 7{\cdot}6$, $S4 = 8{\cdot}5$, F.H. = 16·9

(*b*)

	H	*L*
*K*1 . .	21·9	5·4
*K*3 . .	8·9	5·1
*K*5 . .	4·2	3·6

(*c*)

	*K*1	*K*3	*K*5
A . .	10·9	6·1	2·9
C . .	14·1	8·4	5·2
M . .	16·0	6·6	3·7

(*d*)

	*S*1	*S*2	*S*3	*S*4	F.H.
*K*1 . .	4·7	7·6	12·7	14·5	28·8
*K*3 . .	3·0	3·9	6·1	7·7	14·5
*K*5 . .	2·3	2·5	4·2	3·3	7·3

The discussion of the data presented will be deferred to the second part of this paper, in which further growth data will be presented and a comparison made of the nutrition requirements of bracken and phanerogamic plants.

SUMMARY

1. Methods were evolved for growing large numbers of bracken sporelings of uniform size. They involved partial sterilization of spores by washing and several transplantings of prothalli and sporelings; except for the initial sowing of spores on agar, sand culture was employed. A water-culture method was also developed and is described.

2. Some general observations on the growth of the bracken plant from the spore to the mature sporophyte are presented.

3. Preliminary experiments on the mineral nutrient requirements of the prothallus indicated the importance of adequate phosphorus and nitrogen supply. Potassium and sulphur deficiencies also caused symptoms.

4. The design of the main experiment of the 1946 season on sporophyte nutrition consisted of a factorial combination of 3 potassium levels × 2 phosphorus levels × 3 solution types. The solutions differed in having their anions supplied mainly as Na salts (*A*), mainly as Ca salts (*C*), or mainly as NH_4 salts (*M*). Four samples were collected during the growing period as well as a final harvest.

5. Phosphorus and potassium deficiency symptoms in the three basal nutrient media are described.

6. Rates of leaf production and death were determined from weekly leaf counts, and the average longevity of leaves in different treatments calculated from these. Production was severely reduced by P and K deficiencies, while longevity was much decreased by K deficiency.

7. Numbers of leaf-buds and rhizome branches counted at sampling times are presented. Statistically significant effects include a rise in leaf-bud numbers at the end of the season when leaf expansion has declined. Significant interaction effects between nutrients, basal solution, and age were also found.

8. Discussion of the data has been deferred to the second part of the paper in which the bulk of the growth data will be presented.

Acknowledgements

The author is much indebted to Professor F. G. Gregory for suggesting the investigation and for his inspiration and encouragement throughout the experiment. In particular his thanks are due to Mr. F. J. Richards for his unfailing help and advice as well as his kindly criticism at all stages of this work. Mr. F. J. Levy, F. Last, and A. Wesley kindly helped at the sampling occasions, and Dr. N. L. Pal assisted with the starch estimations.

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EXPLANATION OF PLATE XVIII

Illustrating W. W. Schwabe's article on 'Physiological Studies in Plant Nutrition, XVI. The Mineral Nutrition of Bracken, Part I. Prothallial Culture and the Effects of Phosphorus and Potassium Supply on Leaf Production in the Sporophyte'.

A. Degree of differentiation of primary sporeling leaf as determined by the size of the mother prothallus.

B. Leaf area of *HCK*1 plant at sample 2.

A. Degree of differentiation of primary sporeling leaf as determined by the size of the mother prothallus

B. Leaf area of HCK_1 plant at sample 2

W. W. SCHWABE

The Fixation of Nitrogen associated with the Root Nodules of *Myrica gale* L., with Special Reference to its pH Relation and Ecological Significance

BY

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With Plate XIX

Abstract

The paper deals with the fixation of nitrogen by nodulated plants of *Myrica gale* under experimental conditions, the fixation being, in the first year of development, of a magnitude comparable to that of legumes under similar conditions. The effect of acidity on the fixation has been investigated. The *Myrica* organism is markedly adapted to acid conditions. The observations are correlated with field data in Britain and help to explain the distribution and habits of the species.

Introduction

According to van den Bergh and van Vloten (1928) *Myrica gale* L. (Bog Myrtle or Sweet Gale) occurs in most north-western European countries, particularly in coastal areas, while Youngken (1919) records its occurrence along with other *Myrica* species in North America. In Scotland it is a dominant plant over large areas of wet acid peat soils.

The physiological significance of the nodules regularly present on the roots of *Myrica* spp. had received little attention until Bond (1949) presented preliminary evidence that nitrogen fixation is associated with the nodules of *M. gale*. In the present paper more extensive data in support of this are presented, and the ecological importance of the fixation is considered. In addition, observations on the pH relation of the development of the nodules and of the fixation process in water culture are reported. From consideration of the typical habitat of *M. gale* it appears that the endophyte and the nodules are much more tolerant of acid conditions than in the legume. Experimental observations of the type indicated, besides illustrating the full extent of this difference in pH relation, seemed likely to be of interest ecologically in giving

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greater understanding of the distribution of *M. gale* in the field. Quantitative data regarding non-leguminous symbiotic fixation of nitrogen are in general scanty, and there has been little progress beyond the mere establishment of the fixation. It is hoped to remedy this deficiency for other non-legumes, besides bog myrtle, by means of work in progress in this department.

The question of the nature of the endophyte of the nodules of *M. gale* will not (except in one connexion) be considered in this paper, but it may be noted now that while, as in other non-legumes, a number of investigators of the cytology of bog myrtle nodules have concluded that the endophyte is an Actinomycete, others have taken the view that it is fungal, bacterial, or myxomycetal in nature, or that two organisms are present. This uncertainty is partly due to the fact that no confirmed isolation of the endophyte has yet been achieved for a non-leguminous root nodule.

Methods

Seed collected from the field in the previous autumn was sown in early spring in trays of horticultural peat which were placed in a cool greenhouse. Prior to sowing, the seed had been stored for several weeks in similar peat at a temperature of +2° C. Evidence of the favourable effect of this pre-treatment is presented in the next section. The peat used for the above purposes was not sterilized and was moistened with tap-water; water extracts of it gave a pH of 3·5.

Seedlings were transplanted into water culture when 2 to 4 leaves had been formed, no nodules being present on these plants. For the water cultures, glazed earthenware jars of capacity 2 litres were used, covered by waxed squares of teak ($\frac{1}{2}$ in. thick) bored with 7 holes. Each hole was fitted in its lower region with a $\frac{1}{8}$-in. section of thick-walled rubber tubing, the diminutive seedling being supported in the central hole of this by a small rubber wedge.

The basic culture solution employed was a nitrogen-free form of Crone's solution made up according to the following formula, which agrees with the original formula except for the substitution of KCl for KNO_3:

KCl	0·75 g.
$CaSO_4.2H_2O$ . .	0·50 ,,
$MgSO_4.7H_2O$ . .	0·50 ,,
$Ca_3(PO_4)_2$ . . .	0·25 ,,
$Fe_3(PO_4)_2.8H_2O$ . .	0·25 ,,
Distilled water . .	1 litre

Where plants were to be supplied with combined nitrogen, appropriate amounts of $NaNO_3$ were added periodically to the culture jars containing the above solution. One cubic centimetre of a minor element concentrate, based on Hoagland's A to Z solution (Templeman, 1941) with molybdenum added, was supplied to each litre of the Crone's medium. The pH of the above solution was 6·3, and one series of plants was supplied with the solution at this pH. For other series the solution was previously adjusted to pH 5·4,

4·2, and 3·3 respectively by suitable addition of sulphuric acid. The culture solution in the jars was replaced at intervals of approximately 3 weeks, during which the pH in jars containing nodulated plants showed little change, but in the later stages of the experiment it became necessary to readjust pH every 2 days in cultures supplied with nitrate-nitrogen.

Inoculation of plants with the nodule organism was effected 24 to 48 hours after transplanting by applying to each root system several drops of a suspension in water of pulverized nodules from field plants. A similar amount of an autoclaved suspension was added to jars in which nodulation was not desired.

Most of the plants were grown for one season only, and were harvested when leaf-fall was about to commence. Each plant was harvested separately, dry weights being obtained by heating at 95° C. and total nitrogen by Kjeldahl process. A few plants were allowed to continue growth for more than one season, and were transplanted into individual 8-litre jars at the commencement of the second season.

Data

Observations on germination

The percentage germination of seed collected in the autumn and sown in the following spring after storage at room temperature proved to be low. Experience showed that the germination could be considerably improved by storing the seed in moist peat at low temperature for several weeks prior to sowing. It was subsequently noted that this agreed with the findings of Barton (1932) for *Myrica carolinensis*. As an illustration, the results of tests in 1949 on two samples of seed of *M. gale* are presented below, the seedlings being counted 8 weeks after sowing, by which time germination appeared to be practically completed.

	With prior storage of seed at room temp.			With prior storage for 11 weeks at +2° C.		
Sample.	No. sown.	No. of seedlings.	% germination.	No. sown.	No. of seedlings.	% germination.
A	1,800	33	1·8	1,800	275	15·3
B	750	60	8·0	1,500	200	13·3

It is possible that the greater germination given by sample B seed stored at room temperature compared with corresponding seed of sample A was due to the fact that the former seed was not collected until January, while sample A was collected in September, before there had been any natural exposure to low temperatures. Storage of seed at −8° C. was found to be less beneficial than that at +2° C.

Water-culture experiments

Two experiments involving the study of the growth of plants in water culture at different pH levels are reported in this paper. The first, carried

out in 1949, comprised inoculated and control plants, the seedlings being set up in water culture on May 28. The second was performed in 1950 and included plants as above and also non-inoculated plants supplied with nitrate-nitrogen. Here the seedlings were transplanted into water culture on May 6.

The progress of the *inoculated plants* in both experiments can be considered together. At pH 6·3 and 5·4 nodules became visible to the naked eye 2 to 3 weeks after inoculation, and at this stage formed bright red swellings on the roots. Nodulation was slower and more sparse in the more acid solutions. After 4 weeks' growth there was already evidence of nitrogen fixation in that the more abundantly nodulated plants showed stronger top growth than the uninoculated controls. This superiority became accentuated as time went on.

The paucity of nodule formation at pH 4·2 and 3·3, particularly in the latter, was accompanied by poorer general growth. A number of plants failed to form any visible nodules, became moribund, and eventually died. Some loss of plants in this way occurred in all solutions, but was much greater with increasing acidity, as will be confirmed by data below.

The red pigmentation of the young nodules was gradually replaced by a buff colour, and by the end of August few nodules were still red. The rootlets which in *Myrica* spp. develop from the tips of the nodule-lobes showed strongly upward growth and soon projected from the surface of the culture solution (Bond, 1949).

In both experiments the nodulated plants were harvested at the end of October, i.e. when they were 5 to 6 months old. All plants still living were included in the harvest, the mean data being presented in Table I, together with statistical treatment. The results of the two experiments agree in their main features. The mortality among the plants is indicated by deduction of the number harvested from the number initially transplanted, and in this connexion the 1950 data give a truer picture of the position, since in the previous year plants dying-off at an early stage were replaced by new ones. It will be seen that only one plant survived at pH 3·3 in 1950.

In both years the highest mean values for height, dry weight, and nitrogen content were obtained at pH 5·4. It has to be noted, however, that at each pH there was substantial variation in size between individual plants, as is indicated by the 'range' figures for dry weight and nitrogen content shown in the table. This variation in capacity for growth is presumably due in the main to the original seed being of wild origin and from an unselected population. Its effect, coupled with the poor survival rate at lower pH, is that full statistical significance for differences between means is not always attained (see table). The analysis of variance also indicated that in respect of total dry weight in 1949 and total nitrogen in 1950 the over-all effect of pH just failed to reach significance at the $P = 0{\cdot}05$ level, but in view of the general consistency of all the data there is no reason to doubt that the picture of the pH effect presented by the experiments is substantially correct.

Photographs of typical nodulated plants from these experiments are shown in Pl. XIX, Fig. 2. In plants of this age the nodules are concealed by the dense

TABLE I

*Mean Data obtained at Harvest of Nodulated plants**

Year.	pH of culture solution.	No. of plants harvested.†	Height of shoot (cm.).	Dry weight per plant (mg.). Nodules.	Top and root.	Total.	Total nitrogen per plant (mg.).
1949	3·3	8	8	46 (12–129)‡	422	468 (122–1,249)‡	13·3 (2·8–35·0)‡
	4·2	8	12	68 (16–128)	771	839 (226–1,258)	23·6 (7·8–33·4)
	5·4	11	15	85 (13–129)	1,045	1,130 (358–2,109)	32·7 (10·7–67·8)
	6·3	15	9	48 (12–139)	601	649 (132–2,029)	17·9 (14·2–53·3)
1950	3·3	1	10	25	355	380	11·0
	4·2	16	14	57 (10–171)	701	758 (165–2,191)	23·1 (5·6–62·7)
	5·4	22	17	81 (24–162)	1,153	1,234 (429–3,087)	33·6 (12·0–78·1)
	6·3	31	15	52 (13–117)	908	960 (253–2,277)	26·2 (6·9–57·9)

* In each year approximately 8-week-old seedlings were transplanted into water culture in May, harvest being effected in the following October.

† In 1949, 28 plants were initially set up at each pH (but see text regarding replacement of plants dying-off). In 1950, 35 plants were set up at the three lower pH levels, and 42 at pH 6·3.

‡ The figures in parentheses indicate the lowest and highest individual values.

Minimum Differences between Means required for Significance at $P = 0{\cdot}05$, from analysis of variance

	Comparison.	No. of plants.	Dry weight (mg.).	Total nitrogen (mg.).
1949	pH 4·2 and 3·3	8 and 8	566 (obsvd. 371)	13·7 (obsvd. 10·3)
	pH 5·4 and 4·2	11 and 8	527 (291)	12·7 (9·1)
	pH 6·3 and 5·4	15 and 11	451 (481)	10·9 (16·8)
1950	pH 5·4 and 4·2	16 and 22	379 (476)	10·3 (10·5)
	pH 6·3 and 5·4	31 and 22	322 (274)	8·7 (7·4)

growth of rootlets which, as noted, develop from the nodules themselves. Nodules are, however, visible in the photograph of a younger plant included in the preliminary account (Bond, 1949).

No indication of the number of nodules is given in the table, for the reason that the originally simple nodules soon develop into complex branched structures which present no unit portions convenient for counting. The mean figure for nodule dry weight, expressed as a percentage of the dry weight of the whole plant, amounted to 7·4 per cent., which is of the same order as observed in legumes under similar growth conditions. The nodules showed a mean nitrogen content of 3·4 per cent., compared with 2·8 per cent. for the plant as a whole.

Reference to the data for the *uninoculated control plants* (all of which remained nodule-free) in Table II indicates that negligible amounts of combined nitrogen were available to the plants from unintended sources. Thus practically all the nitrogen accumulated in the nodulated plants (Table I) is concluded to be the result of fixation. The highest fixation was shown by a plant at pH 5·4 in the 1950 experiment and amounted, as indicated in the table, to 78 mg. during the growth period of 6 months. Pl. XIX, Fig. 1, illustrates for pH 5·4 the relative sizes of nodulated and control plants.

TABLE II

*Mean Data obtained at Harvest of Uninoculated Control Plants**

Year.	pH of culture solution.	No. of plants.	Height of shoot (cm.).	Dry weight per plant (mg.).	Total nitrogen per plant (mg.).†
1949	6·3	14	2	16‡ (12–21)	0·4 (0·3–0·6)
1950	3·3	7	1	8	0·1
	4·2	7	1	7	0·1
	5·4	7	1	10	0·2
	6·3	21	1	8	0·2

* These plants were set up in water culture on the same date as the inoculated plants (Table I), but owing to what were obviously conditions of extreme nitrogen-deficiency few of them survived the full experimental period. In view of this plants were harvested at intermediate dates as they became moribund.

† The mean nitrogen content per original seed = 0·04 mg.

‡ The higher dry weights and nitrogen contents shown in 1949 are due to the plants having been rather larger when they were transplanted from peat into water culture than in 1950.

Turning to the *uninoculated plants supplied with nitrate-nitrogen*, the first addition of nitrate to these plants was made 1 week after transplanting, when the equivalent of 100 mg. nitrogen was added per jar. Three weeks later the plants in the more acid solutions had become noticeably stronger than those at higher pH, a tendency to chlorosis being shown in the latter. This was not relieved by the addition of appropriate amounts of ferric citrate; that the chlorosis was not due to iron-deficiency was confirmed by the fact that similar symptoms were not evident in the corresponding nodulated plants. This chlorosis, especially marked at pH 6·3, continued throughout the experiment, and was accompanied by a marked tendency for plants to die off (see also below). It appears that at this relatively high pH the plants were unable to utilize the nitrate-nitrogen.

As noted already, in the later stages of growth of these plants a frequent adjustment of the pH of the culture solution in the jars became necessary, because of a tendency for pH to rise rather rapidly, especially with the more vigorous plants in the lower pH series. Thus rises of 0·5 to 0·7 of a pH unit were sometimes shown over 48 hours. This tendency is of course frequently experienced in culture experiments where nitrate-nitrogen is being utilized.

The plants were harvested at the end of August, when leaf-fall showed signs of approaching. This was 2 months earlier than leaf-fall in nodulated plants, in agreement with an observation made by Roberg (1934) on alder. The data obtained at harvest are presented in Table III, along with statistical analysis. It will be observed that the maximum growth was obtained at pH 3·3. The analysis of variance showed that the over-all effect of pH was highly significant, and although the differences in mean dry weight and nitrogen content between pH 5·4 and 4·2 fail to reach significance (see table), the general conclusion that as pH rose growth decreased is not in doubt. The mortality of plants increased at higher pH. These findings are almost exactly opposite to those for the nodulated plants. The mean percentage nitrogen content of the nitrate-fed plants was 2·7; typical plants are shown in Pl. XIX, Fig. 3.

TABLE III

*Mean Data obtained at Harvest of Non-nodulated Plants supplied with Nitrate-nitrogen**

pH of culture solution.	No. of plants initially set up.	No. of plants harvested.	Height of shoot (cm.).	Dry weight per plant (mg.).	Total nitrogen per plant (mg.).†
3·3	21	14	23	1,004 (375–1,720)‡	28·9 (11·2–50·5)‡
4·2	21	11	16	703 (130–2,230)	18·5 (3·4–64·1)
5·4	21	11	16	565 (155–1,885)	15·4 (3·7–59·0)
6·3	21	8	9	90 (20–262)	2·3 (0·6–7·4)

* The growth-period in water culture for these plants extended from May to August 1950.

† A total of 150 mg. nitrate-nitrogen per plant was supplied to the culture solution during the experiment.

‡ The figures in parentheses indicate the lowest and the highest individual values.

Minimum Differences between Means required for Significance at $P = 0{\cdot}05$, from Analysis of Variance

Comparison.	No. of plants.	Dry weight (mg.).	Total nitrogen (mg.).
pH 4·2 and 3·3	14 and 11	381 (obsvd. 301)	11·4 (obsvd. 10·4)
pH 5·4 and 4·2	11 and 11	403 (138)	12·1 (3·1)
pH 6·3 and 5·4	11 and 8	439 (475)	13·2 (13·1)

No nodules developed on these plants. Comparing the data, it is seen that the largest of the plants receiving nitrate were somewhat smaller than the best nodulated plants. Catkin buds were present on several nodulated and nitrate plants at harvest, some plants bearing male and other female buds. An older nitrate-fed plant, photographed in its second season, is shown in Pl. XIX, Fig. 4.

Discussion

Fixation in water culture

The results presented above show the ability of nodulated plants of *M. gale* to make vigorous vegetative and reproductive growth in culture solution free of combined nitrogen, and thus confirm the evidence for fixation given in the preliminary report (Bond, 1949). As noted, the maximum fixation under the conditions prevailing amounted to 78 mg. nitrogen per plant during the first growth season. This is comparable with the fixation shown by annual legumes such as pea or Soya bean under similar conditions. A few nodulated plants of bog myrtle have been allowed to continue growth for longer periods, still in nitrogen-free solution.[1] Pl. XIX, Fig. 5, shows such a plant in its third season. This plant, when harvested at the end of that season, had 30 shoots which ranged up to 85 cm. in length, while the total dry weight of the plant was 201 g. and its total nitrogen content 3·5 g. The plant was female and bore a quantity of ripe fruit. These data are considered further below.

Strong as the evidence for fixation is, it is desirable that there should be eventual confirmation under aseptic conditions, since in the present experiments there is the possibility, certainly very remote, that a free-living nitrogen-fixing organism was introduced into the culture jars with the inoculum, and that products of the fixatory activity of this organism rather than that of the nodules accounted for the strong growth of nodulated plants.

It is evident, assuming that the fixation of nitrogen occurs in the first instance in the nodule, that some mechanism provides for the rapid transfer of the bulk of the fixed nitrogen from the nodule to the plant as a whole, as in the legume (Bond, 1936; Jensen, 1948).

Effect of pH

The data on the effect of the pH of the culture solution on growth presented in the previous section may be summarized as follows:

(*a*) Nodulated plants grew best at pH 5·4, though substantial growth and fixation were also shown at pH 6·3 and 4·2. At pH 3·3 many plants failed to form nodules and soon died; one of the plants which did form nodules at this latter pH grew, however, fairly strongly (Table I, 1949 data).

(*b*) Non-nodulated plants supplied with nitrate-nitrogen gave almost opposite results, since they grew best at pH 3·3 and with increasing pH showed diminishing growth, extremely poor plants being obtained at pH 6·3.[2]

[1] The solution was supplied at pH 6·3, but tests showed that with these older and larger plants the pH fell rapidly to values in the range 3·5 to 4·5, so that for most of the growth period a pH of the latter order prevailed.

[2] This harmful effect of relatively high pH does not seem to apply to older plants dependent on nitrate-nitrogen. Thus a plant which at the time of writing is at the end of its second season, and which for the first 2 months was grown at pH 5·4, has for the last 18 months been supplied with solution at pH 6·3. Under the influence of the plant pH has risen as high as 7, but satisfactory growth has been shown (see Pl. XIX, Fig. 4).

Further experiment might well show that if supplied with ammonium-nitrogen non-nodulated plants would grow well at the higher pH levels, but the present data establish the point that given a suitable source of nitrogen the bog myrtle plant is able to grow vigorously at pH 3·3. The failure of inoculated plants to do likewise is clearly due to the deterrent effect of this degree of acidity on the nodule organism in the culture solution, or on the process of infection, rather than on the development of the plant itself.

In comparison with inoculated legumes, the pH relation of which was reviewed and studied by Jensen (1943), it may be stated, however, that inoculated plants of *M. gale*, with optimal growth at pH 5·4, still vigorous growth at 4·2, and occasionally appreciable growth at pH 3·3, have much greater aptitude to acid growth conditions. This is due to the relative ability of the endophyte to withstand an acid medium, and to the capacity of the plant itself for vigorous growth in such a medium.

Ecological aspects: nitrogen fixation in the field

Pearsall (1938) records 4·3 to 5·6 as the typical pH range for *M. gale* in the bogs (mostly in northern England) studied by him, with an extension of the lower limit to 3·8 in some areas. The examination of the pH of 26 soil samples from bog myrtle stations in western Scotland by the present author yielded a mean value of 4·2, with a range of 3·7 to 4·8.[1] In these latter areas it appears, on the basis of the water-culture experiments, that the species is growing at pH levels somewhat below the optimum for nodulated plants, and at which a considerable mortality of seedlings was experienced in nitrogen-free culture owing to non-development of nodules. It is possible that here, coupled with the low viability of seed, we have the explanation of the rare occurrence of seedling plants in the areas studied by the author, where the species spreads almost entirely by means of offshoots arising underground from established plants. The infrequency of bog myrtle in areas where pH is lower than 3·8, suggested by the available data, could obviously also be explained by the water-culture results, and points to a high degree of dependence of the plant on nodule-nitrogen in the field, since given a source of nitrogen the plant itself could thrive at lower pH.

There seems no reason to doubt that the fixation demonstrated for cultured plants also proceeds in the field, and from the data given on p. 454 relating to the growth of an older nodulated plant in culture it is possible to make a provisional estimate of fixation in the field. Of the 3·5 g. nitrogen fixed by the plant in its first 3 years, it is reasonable to assume that 2·5 g. was fixed in the last of these years. If this quantity is divided by the number of larger shoots (over 50 cm. in length) present by the end of that year, namely 20, then a fixation of 125 mg. nitrogen per shoot is indicated. Assuming a density of 6 shoots per square yard in the field, calculation points

[1] The soil samples were collected in September, were air-dried, pounded and sieved, after which 16 g. portions were shaken with 80 c.c. distilled water for 1 hour and pH readings taken in the suspension with the glass electrode.

to a fixation of 8 lb. nitrogen per acre per season. This figure, which is probably a conservative one, falls well below corresponding estimates for leguminous crops, which have varied from 30 to over 100 lb. nitrogen per season (Jensen, 1950). These refer, however, to a more or less pure stand of legumes, and a fairer comparison would be against more natural communities such as legume-grass pastures. The fixation there per acre would obviously be a good deal nearer to the bog myrtle figure.

Bog and moorland soils are generally stated to be typically low in available nitrogen, but it is obvious that there is, in the plant communities occupying these areas, a considerable amount of combined nitrogen in circulation. The origin of this has been obscure, for none of the regular nitrogen-fixing systems is likely to function, either because of their absence from such areas, as applies to legumes, or because even if present the low pH would according to the available evidence preclude fixation. Boswell and Gover (1946) isolated a *Clostridium* and an *Azotobacter*-like organism from a soil of pH 3·3, but they do not envisage any fixatory activity in such soils. At least in those areas where *M. gale* occurs the fixation associated with it can be concluded to have considerable ecological importance. By such drainage as occurs the benefit of the fixation will be extended to adjacent tracts of land or of fresh water. It is clear that symbiotic systems have an advantage under conditions of unfavourable acid medium in that within the tissues of the host plant the endophyte is likely to find a more appropriate pH for the fixation process. There are, of course, other symbiotic associations, such as those of the Ericaceae, occurring in habitats related to those favoured by *M. gale*, for which a function of nitrogen fixation has been suggested, though not yet adequately confirmed.

Miscellaneous aspects

The conclusions which may be drawn regarding the bog myrtle endophyte from these experiments are as follows. The organism is present in or on nodules from the field in a form capable of producing infection of new plants, and the infection can take place in an aqueous medium over a pH range of 3·3 to 6·3, though it is greatly curtailed at the lower end of this range.[1] The organism is probably not seed-borne since seedlings do not develop nodules unless deliberately inoculated, though it must be noted that the low pH of the peat used for germination would tend to deter nodulation even though the endophyte were present.

The red pigmentation of young nodules is a conspicuous feature of plants in water culture, but has not been detected by the author in field material to anything like the same degree, though a similar red pigmentation is prominent in underground shoots in the field. Previous authors, working

[1] As noted in the Introduction, several investigators of nodule cytology have concluded that the *Myrica* endophyte is an Actinomycete; though organisms of this group typically prefer alkaline media, Jensen (1928) has found strains in acid peat soils which showed optimum growth at pH 3 to 4.

with field material, have correctly described nodules as being yellowish-brown in colour, though Arzberger (1910) and Youngken (1919) mention a pink colour in nodules of *M. cerifera*. Young nodules of alder in water culture also show an intense red colour, as noted by Krebber (1932) and confirmed by the present writer. The similarity in colour between these non-legume and legume nodules is obviously coincidental, since it is clear that the pigment concerned in the bog myrtle nodule is of anthocyanin type.[1] Thus sections of the nodule show intensified redness in dilute acid, while in alkali a blue colour is assumed; larger-scale tests with underground shoots from field material were all indicative of anthocyanin. Since the pigment does not persist for very long in the nodules it seems unlikely to have any function in connexion with fixation. Its initial development may be connected with the particularly high carbohydrate/nitrogen ratio in young plants transplanted into nitrogen-free solution. A pigment of similar hue frequently appears in the roots of water-culture plants, particularly if they are exposed to light.

The strong upward growth of the rootlets developing from the apices of the nodule lobes is a very curious feature of nodulated root systems of *M. gale* in water culture. A tuft of such rootlets is to be seen at the base of the stem in the nodulated plants of Pl. XIX, Figs. 1 and 2. This feature is also shown to a lesser extent by the lateral roots of non-nodulated plants receiving nitrate-nitrogen, and will form the subject of a later paper.

Summary

Further evidence is presented to show that fixation of nitrogen is associated with nodulated plants of *Myrica gale* under experimental conditions. During the first growth season of young plants the fixation per plant was of comparable magnitude with that of legumes under similar conditions.

When young plants, growing in water culture without combined nitrogen at four different pH levels in the range 3·3 to 6·3 were inoculated with the nodule organism, nodules formed at all pH levels but were sparse at pH 3·3, and most of the plants at this pH died. The best growth of nodulated plants during their first season was at pH 5·4.

Non-nodulated plants supplied with nitrate-nitrogen showed strongest growth at pH 3·3, indicating that the poor growth of inoculated plants at this pH was due to difficulties in nodule formation, and not to the inability of the plant itself to grow under such acid conditions. Compared with the legume nodule organism the *Myrica* organism, however, is markedly adapted to acid conditions.

These observations show satisfactory correlation with data on the pH relation of *M. gale* in the field, and help to explain the distribution and habits of the species.

[1] After the publication of the preliminary report (Bond, 1949), Dr. J. D. Smith, of the Molteno Institute, Cambridge, requested nodule material for spectroscopic examination. The present writer was informed that no evidence of the presence of haemoglobin was obtained.

There is no reason to doubt that fixation of nitrogen is also associated with the normally nodulated plant in the field, and that the fixation is of considerable ecological importance in the bog habitats frequented by *M. gale*.

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EXPLANATION OF PLATE XIX

Illustrating G. Bond's article on 'The Fixation of Nitrogen associated with the Root Nodules of *Myrica gale* L., with Special Reference to its pH Relation and Ecological Significance'.

Fig. 1. Plants grown for 20 weeks in water culture without combined nitrogen, 1950 experiment, pH 5·4; on the left, a control plant without nodules, on the right, a nodulated plant. ($\times \frac{1}{7}$.)

Fig. 2. Nodulated plants of 1950 experiment after 20 weeks' growth in water culture without combined nitrogen, the pH of the solution in which the plants were grown being (from left to right) 3·3, 4·2, 5·4, and 6·3 respectively. The plants were selected as being of average size. ($\times \frac{1}{7}$.)

Fig. 3. Non-nodulated plants supplied with nitrate-nitrogen, 1950 experiment, after 16 weeks' growth in water culture at pH (from left to right) 3·3, 4·2, 5·4, and 6·3 respectively. The plants were selected as being of average size. ($\times \frac{1}{8}$.)

Fig. 4. A single non-nodulated plant supplied with nitrate-nitrogen, transplanted as a seedling into water culture in May 1949 and photographed August 1950. For details of pH see footnote to p. 454. ($\times \frac{1}{15}$.)

Fig. 5. A single nodulated plant transplanted as a seedling into water culture in May 1948 and photographed September 1950. For details of pH see footnote to p. 454. ($\times \frac{1}{15}$.)

G. BOND

An Electron Microscope Study of the Spermatozoid of *Fucus serratus*

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With Plates XX–XIV

Abstract

A detailed description with plates is given of the flagellar structure and cell morphology.

THE evidence contained in the plates accompanying this paper amplify the preliminary communication published in 'Nature' (Manton and Clarke, 1950) with respect to the morphology and structure of the various appendages of the spermatozoid of *Fucus*, to which some information on the contents of the body is added. In this last respect the *Fucus* male cell has shown itself to be unusually amenable to study with the electron microscope owing partly to its exceptionally small size among plant motile cells. Under suitable conditions, as Pl. XXIII will show, the body becomes transparent even to the very limited powers of penetration of the electron beam in a way which is not often obtainable even with bacteria. Not everything which is revealed by this means is interpretable. The task of collating the new evidence with previous knowledge will, however, perhaps be facilitated by the low-power views of whole cells both normal and abnormal, which are assembled on Pl. XX, together with some high-power ultraviolet photographs of similar material examined directly in sea-water without either staining or drying (Figs. 1–3). These represent the most natural pictures which can be obtained with the light microscope, and though necessarily less perfect in details than the electron micrographs, they provide a valuable means of assessing the extent of distortion which is entailed in preparing the specimen for electron microscopy.

Material and Methods

Sources of material need little explanation. Male plants of *F. serratus* have been obtained by post at various times of the year from both east and west coasts, the western source being the Welsh coast at Aberystwyth and the eastern source being various places along the Yorkshire and Durham coasts, from some of which material was brought back to Leeds by hand. Except in hot weather there seems no disadvantage in sending material by post provided it is closely packed and kept moist but without free water. On arrival at its destination it should be stored in a refrigerator without being unpacked, and

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spermatozoids can then be hatched out at will over a period of several days if fertile tips are merely put into fresh sea-water at or below room temperature. When conditions are right such water will almost at once become a swarming mass of motile cells without many dead ones between. If only a few motile cells are obtained the material is unsuitable.

Thirty seconds' exposure to the vapour from 2 per cent. osmic acid was the killing agent used throughout this study both for ultraviolet microscopy and for electron microscopy. This can be done directly on a quartz slide for the ultraviolet microscope or on a formvar carrier for the electron microscope if the material is to be examined at once. Immediate examination is essential if the object of study is the internal contents of the body or the fibrillar disintegration of the cilia. For gross morphology, storage in formalin diluted to 8 per cent. with sea-water is convenient and by hardening the various parts can be a definite advantage over fresh material for certain purposes such as the study of the hairs on the front flagellum.

The other technical details are those customary in work with these two instruments. For ultraviolet microscopy the material on a quartz slide is merely covered with a quartz coverslip and ringed with wax to prevent drying. For electron microscopy material is dried on to the formvar carrier. Unwanted debris and salts may have to be removed by washing either before or after the initial drying down, or both. Shadow casting is then carried out on all specimens other than those required for internal contents of the body, the metal used in this particular study being uranium.

The ultraviolet microscope used is the Cooke, Troughton and Simms instrument working with Zeiss quartz lenses computed for the 2750A Cadmium line. The electron microscope is a Philips instrument. Both are in the Botany Department of Leeds University.

The Ultraviolet Photographs (Plate XX, Figs. 1–3.)

Owing to the very shallow depth of focus possessed by all high-power lenses available with the light microscope it is only possible to photograph sample optical sections, not all parts of which will be equally in focus (Figs. 2 and 3), or else to assemble several different exposures at different focal levels through the same specimen if all parts of it are to be even roughly seen (Figs. 1*a–c*). This limitation would excite no comment in work involving only the light microscope, but this particular difficulty is so completely absent from electron micrographs that it is perhaps necessary to refer explicitly to it or certain parts of Figs. 1–3 may perhaps be misunderstood.

Figs. 1*a–c* represent three different focal levels through two spermatozoids, the upper one of which has only just left the antheridial wall and is still motionless, having not yet uncoiled its various appendages, while the lower one is in the fully motile condition. As this cell, together with those of Figs. 2 and 3, will show, the front flagellum is slightly longer than the asymmetrical body, while the back flagellum (Fig. 1*c*) is about three times the length of the body and rather more than twice the length of the front flagellum.

The large dark object visible inside the body in Figs. 1*b* and *c* is not the nucleus but the eyespot, which appears orange when seen by visual light. In position, the eyespot lies on the flattened side of the asymmetrical body between the points of emergence of the two flagella. These can be seen best in the silhouette view of Fig. 2. Though they come out of the body a considerable distance apart they can be traced inside the body with both the ultraviolet microscope and by the use of stains. A good summary of existing knowledge in this respect will be found in the figures reproduced by Fritsch (1945, p. 375) or in the excellent early accounts published by Guignard (1889) and Meves (1918). The hind flagellum passes actually across the surface of the eyespot inside the body before leaving it and in the forward direction it joins on to the base of the front flagellum at the 'blepharoplast' or basal granule. This organ will be seen very clearly indeed in the electron micrographs which follow, and since it can only be adequately demonstrated in the light microscope by the use of stains, it is not shown in the ultraviolet photographs.

Some other cell inclusions are shown. Small granules such as that on the lower side of the left-hand cell of Fig. 3 which absorb ultraviolet strongly are probably metabolic products as described by several observers (e.g. Retzius, 1905; Meves, 1918). The nucleus is, however, rather unusually difficult to see. It does not absorb ultraviolet appreciably more intensely than the cytoplasm and its reaction to stains, to judge by the literature, seems equally weak. The affinity for nuclear stains found in the cytoplasm has, indeed, led a number of observers (Retzius, 1905; Meves, 1916; Kylin, 1920, but not Kylin, 1916) to the view that almost the whole cell body was in fact of nuclear origin. That this view is incorrect seems demonstrated here by the clarity with which the electron beam picks out differences in density and in so doing reveals the nucleus as a fairly large body within the cell but not occupying the whole of it and placed immediately behind the eyespot. The clearest view of what is almost certainly the nucleus in the ultraviolet photographs is in the left-hand cell of Fig. 3. In this the eyespot is out of focus and appears in the middle of the cell as a pale ring with a dark centre. Extending backwards from it, the outline of a larger body is visible which touches the metabolic granule previously referred to on its lower side but which extends backwards almost to the hinder end of the cell. This, allowing for reduction in size by shrinkage on drying, agrees sufficiently well with the electron-microscope evidence to be accepted as the same organ.

Lastly the proboscis. This organ can be seen very easily after chemical killing as a translucent structure of rather indeterminate shape curving away from the base of the front flagellum on the body side of it. It can be seen in Fig. 1*c*, Fig. 2, and on the right-hand cell of Fig. 3. In life it is less conspicuous owing to a much closer physical relation to the flagellum, although with care it can be seen quite clearly even with the visual light microscope.

The Electron Micrographs (Figs. 4–22)

1. *The form of the body*

The effect of shrinkage on the body is at once apparent on comparing Figs. 1*c* and 5. In the dried condition (Fig. 5) the two flagella seem practically unaltered but the body is much reduced in size although substantially unaffected in shape. One significant artifact is, however, shown both by Figs. 5 and 7 or, more clearly, in Fig. 8. All of these show a loop of the hind flagellum which has burst out of the front end of the body as it dried. In contrast, where the flagellum passes over the eyespot it is still retained in its original position in spite of marked signs of tension. This suggests very strongly that in crossing over the eyespot the hind flagellum is also firmly attached to it, a suggestion which is borne out by some of the details assembled on Pl. XXII.

2. *The front flagellum*

The clearest details of the front flagellum are given in Figs. 8 and 9, both of which belong to the cell illustrated in 'Nature' (Manton and Clarke, 1950) and which is shown more complete though in less detail in Figs. 5 and 6. The garniture of hairs along the two sides is its most remarkable feature. At the front end (top of Fig. 9) the hairs arise singly; elsewhere they are produced in tufts of two or three together spaced at regular intervals in a line running down each side of the flagellar axis. The hairs themselves are of very uniform length and show no signs of the jointed structure described in *Pylaiella* (Manton and Clarke, 1951*a*), though in their arrangement they resemble *Pylaiella* very closely. Their attitudes as they lie on the formvar film do not suggest any high degree of mechanical rigidity even after hardening with formalin, but without formalin hardening they are so fragile that they break up and break off on drying, as can be seen by their mutilated condition on the flagella of Figs. 16 and 17, or by the scattered fragments lying on the formvar film in various parts of Fig. 18. Their function is at present unknown.

3. *The proboscis*

General views of this remarkable organ are contained in several of the figures on Pls. XX and XXII, with the best enlarged details in Figs. 8, 16, and 17. In the unflattened or living condition the proboscis appears to be a highly mobile, funnel-shaped membrane partially surrounding the front flagellum and attached to the body at its base. In the flattened condition after drying (Figs. 8, 16, and 17) its free margin is roughly circular with a fimbriated edge and it is traversed by thirteen highly characteristic concentric thickenings. Although the main part of the organ is on the body side of the flagellum its two sides are continued downwards onto the flattened, lower, or flagellar body surface as two transparent bands of thickening which are best described with the contents of the body since they can only be seen with certainty when the body has become transparent. As in the case of the hairy garniture of the

front flagellum, the functional significance of the proboscis and of all its details is at present unknown.

4. *The contents of the body*

Pl. XXII assembles a few characteristic micrographs, Figs. 10–15, of cells which have become transparent to the electron beam. They are all from fresh unhardened cells examined at the highest available voltage. Figs. 10–12 show various views in unflattened specimens, while Figs. 13–15 are from cells which have become partly decomposed and spread upon the formvar, thereby adding additional clarity to certain details. For general purposes the most informative views are those of Figs. 10 and 12, in which it will be noticed that Fig. 10 differs from Fig. 12 only in being viewed from the other side. To assist in the interpretation of details the principal organs in Figs. 10, 12, and 13 are labelled, *e* being the eyespot, *n* the nucleus, *b* the basal 'granule', and *s* being two strands connecting the basal granule with other parts of the cell. Further details about the eyespot *e* are contained in Fig. 13, and both Figs. 13 and 14 are important for adding additional clarity regarding the path of the two strands *s*.

The internal structure of the eyespot has not yet been specially studied by us, although some facts about it can be obtained from Figs. 12, 13, and 15. In all of these the organ can be seen, in varying degrees of clarity, to possess a dark rim and a light centre across which the hind flagellum passes in an exactly median line. In addition, Fig. 13 and, less clearly, Fig. 15 show numerous fine lines crossing the clear central region and apparently joining the flagellum to the eyespot rim. It cannot, unfortunately, be known whether these lines represent pre-existing fibrils or whether they are artefacts produced by drying. They confirm, however, fairly strongly the suggestion already made (p. 464) in connexion with the external shape of the cell-body, that the flagellum not merely passes over the eyespot but is in some form of structural attachment to it.

With regard to the strands *s* these are in some way the most interesting organs in the whole body since, although their nature and function can only be guessed at, their position in relation to the blepharoplast suggests that they have something to do with the motion of the flagella and that they may in fact be simple nerve-strands. Their function cannot, unfortunately, be revealed by the electron microscope, but their path in the cell is fairly clearly visible in Figs. 12–14. The backwardly directed strand (Figs. 12 and 13) passes to the rim of the eyespot on the side nearest to the nucleus: whether it thereafter continues into the nucleus has not yet been determined. The forwardly directed strand passes to the base of the proboscis on the same side of the cell, where it seems to end at, or near, the surface. This strand can be seen best in Figs. 13 and 14.

The only other detectable details about the cell-body are certain fine lines visible in Fig. 15, some of which seem to be internal boundaries of cavities containing the cell inclusions and perhaps enlarged by the act of drying,

whilst others seem to be marks on the cell surface connected with the attachment of the proboscis. The most important of these are indicated by arrows. As explained in a preliminary way on p. 464, both sides of the proboscis are prolonged backwards in the form of apparently structureless thickenings on the lower or flagellar surface. One of these thickenings is short and ends at the level of the blepharoplast; it is marked by a single arrow on the upper side of Fig. 15. The other, which is marked by two arrows on the lower side of Fig. 15, extends the whole length of the cell. It can be faintly seen again on the right side of Fig. 12 and in another view as the transparent left-hand edge to the cell of Fig. 11. The coherent texture of these thickenings is indicated by the fact that they can sometimes be seen tearing away from the body but still attached to the proboscis. In contrast, all other parts of the cell surface seem to consist of naked protoplasm without any trace of a membranous covering.

5. *The fibrillar disintegration of the flagella*

The early stages of decomposition which have given extra clarity to Fig. 13, if extended farther, may lead to the total disappearance of the internal organs, the first to become effaced being the strands *s*. On the other hand, a different type of decomposition leading to fibrillar disintegration of the cilia may be highly informative from an entirely different point of view. The nature of the conditions leading to appearances such as those recorded in Figs. 18–22 are not fully known, but since fibrillar flagella are generally found attached to abnormally small and rounded bodies (cf. Fig. 18) and a change of body-shape of this kind can be observed to take place after death of a cell, it seems probable that specimens disintegrated in this particular way are those which were actually dead before the preparation was made and that fibrillar decomposition of the appendages is due either to the macerating action of water on dead cells or of osmic vapour on dead cells.

An early stage in fibrillar disintegration of the front flagellum can be seen in Figs. 21 and 22, while completely disintegrated specimens are represented in Fig. 19 and, on a spectacular scale, in Fig. 18. This last shows two cells side by side in the same field both with their front flagella completely dismembered. The characteristic shrunken spherical bodies can be seen, and the position of the proboscis on each leaves no doubt as to which flagellum is being displayed.

Close scrutiny of the two cells of Fig. 18 will reveal the following main facts. In both there are exactly eleven strands in the most fully dismembered parts. The clearest count is at the distal extremity of the right-hand cell in which all the strands are separately visible, but careful examination of the central region of the left-hand cell will arrive at the same result. All the strands are of very similar length and a central pair is not as easily distinguishable as in other cases, e.g. *Pylaiella* (Manton and Clarke, 1951*a*), though it is almost certainly present and it can be identified with reasonable probability in the right-hand cell by its position and by the rather close proximity of its

two components. On the two sides of the dismembered flagella traces of Flimmer are still detectable, partly by the bases of the hairs which are still attached but also by loose pieces scattered upon the field. A less easily identified component has been deposited as a wavy line just outside the area of field on which the fibrils are spread. Its nature is uncertain since it could either represent portions of a skin which is dismembering or else some third substance, either internal or external to the flagellar axis, which has been precipitated from solution as the specimen dried. That some other component besides fibrils and Flimmer must normally be present seems certain from the very compact nature of the flagellum before disintegration has taken place. There is, however, no direct evidence either in this or the other cases examined by us to indicate how cohesion is normally maintained.

In contrast to the front flagellum which can be encountered fairly easily in the dismembered condition in this material, the hind flagellum has been met with in a similar state only once. This one occasion is illustrated in Figs. 19 and 20. In Fig. 19 a dismembering front flagellum has become bent back on itself and projects towards the left of the picture. The hind flagellum of the same cell leaves the picture on the right-hand side and its continuation is shown in Fig. 20, the dark rod-like object immediately above it in both views being part of an intact hind flagellum of another cell. Compared with the front flagellum the fibrils of the hind flagellum seem thinner, more easily disrupted into rows of granules rather than a coherent thread, and they seem fewer in number. Exact enumeration is difficult on the basis of only a single specimen, but the central region of Fig. 20 suggests approximately 9 and not 11 strands.

Discussion

Probably the most important of the new observations contained in this paper are those enumerated in the last section on fibrillar disintegration of the two flagella. They indicate, on the one hand, that the front and hind flagella may be found to differ not only in function and morphology but in internal construction also. Of greater interest, on the other hand, are the comparisons with other organisms which are beginning to become possible. It might be expected that a Flimmergeissel would be a rather unique organ with few points in common with other ciliary types outside the particular plant groups (of algae, fungi, and flagellates as enumerated in the literature list of Manton and Clarke, 1950) in which it occurs. This is, however, not the case. Not only is there close resemblance to the numerical facts already published for *Pylaiella* (Manton and Clarke, 1951*a*), a fact which would cause no surprise, but the same is true of the cilia of a fern (Manton and Clarke, 1951) which is as far removed as can well be imagined, both phyletically and structurally, from the spermatozoid of a brown alga; nevertheless, even more clearly than in *Pylaiella* and in *Fucus*, fern cilia, though completely devoid of hairy appendages, break down into eleven strands, two of which are different from the others and central. Among animals, to quote two early examples at random,

the cilia of *Paramoecium* in America were shown by Jakus and Hall (1947) to be composed of eleven fibrils, and in Australia a very elegant demonstration of eleven strands (nine thicker and two centrally placed thin ones) in the sperm tail of the domestic fowl was published in 1949 by Grigg and Hodge. Certain other numbers have been described in animal sperm tails from time to time by other electron microscopists, e.g. 9 strands in the bull spermatozoid (Bretschneider and van Iterson, 1947), 12 in the ram spermatozoid (Randall and Friedlaender, 1950), 11 and 9 respectively in two species of fish (Rötheli, Roth, and Medem, 1950), &c., nevertheless the number eleven seems to be so remarkably prevalent that some fundamental property of the geometrical relations of fibrils in this quantity seems likely to be involved.

With regard to other aspects of the present material the controversy on the whereabouts and size of the nucleus mentioned on p. 463 needs some, though probably not much, further discussion. In our view the electron micrographs are quite incompatible with the large-nucleus idea first introduced by Retzius (1905) and subsequently adopted by Meves (1918), Kylin (1920), and others. If the entire sperm-body except for small inclusions were composed of the nucleus covered by only a thin skin of cytoplasm, some line of demarcation near the surface of the cell could hardly fail to be detected by the electron beam in those cases (Figs. 10–15) in which the body has become transparent. The complete absence of any signs of discontinuity in the outer protoplasmic layers even when subjected to the very rigorous test of electron microscopy seems to us conclusive evidence that it is not in fact there and that the nucleus is what it appears to be, namely, one of the very dense central inclusions which the electron beam cannot penetrate at all.

Lastly, a word of caution about the limitations of the methods used. The surrealist clarity with which the electron microscope can, in favourable circumstances, reveal structural details must not blind us to its incapacity to provide comparable information on function. We are woefully ignorant on almost every detail of the mode of action of every organ which has been described here, and in many cases we can only guess at the relative importance of an organ from indirect evidence. The proboscis is a case in point. We have micrographs (Manton and Clarke, unpublished) almost indistinguishable from those of *Fucus*, for the related genus *Ascophyllum*, and we have seen a proboscis again with visual light on the rather larger spermatozoids of *Himanthalia*. It therefore seems to be a characteristic of the Fucaceae, although in other brown algae, notably in the zoospores of *Pylaiella* and of *Laminaria* (Manton and Clarke, 1951*a*), it was completely absent. From this one might conclude that the proboscis is an unimportant appendage except as an index of phylogeny, or that it is a part of the swimming mechanism and correlated with the relative shortness of the front flagellum which is also a characteristic of the Fucaceae, or that it has some sensory or copulatory function connected with the oogamous reproductive mechanism in which the spermatozoids, unlike the zoospores, play an essential part. We have no direct evidence as yet on any of these questions, and the only indication that

can be quoted in favour of the proboscis being at least an important organ and not a trivial appendage is the apparently purposeful relation which it seems to bear to the mysterious strands *s* described on p. 465.

It is therefore clear that we are a very long way indeed from possessing a complete descriptive understanding of these very interesting and curiously animal-like plant cells, and it is greatly to be hoped that other methods will be developed to supplement those at present in use.

Summary

The observations recorded amplify the preliminary communication published in 'Nature' (1950).

The hairs on the front flagellum are shown to arise in tufts arranged in two rows down the sides of the flagellar axis. This axis, on disintegrating, breaks down into eleven strands and perhaps some intercalary material. The path of the flagellum through the body has been seen. Front and back flagella make a continuous straight line, but their point of junction is marked by a swelling which seems to correspond to the blepharoplast or basal 'granule' of the light microscopists. The hind flagellum, in passing over the eyespot, seems to be firmly attached to it. The blepharoplast is connected to the edge of the eyespot by a special strand. A similar strand passes forward from the blepharoplast to end at, or near, the surface of the cell on one side of the base of the proboscis. The proboscis is a funnel-shaped membrane traversed by thirteen concentric bands of thickening and its mode of attachment to the body is described. The position of the nucleus is described and discussed.

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EXPLANATION OF PLATES XX–XXIV

Illustrating Manton and Clarke's article on 'An Electron Microscope Study of the Spermatozoid of *Fucus serratus*'.

Note. The magnifications given against electron micrographs are only approximate and should not be used as a basis for detailed measurements.

PLATE XX

Fig. 1. High-power U.V. (2,750A) photograph of a freshly killed spermatozoid mounted unstained in sea-water, three sample focal levels through the same cell to show tip of front flagellum (Fig. 1*a*); eyespot (Fig. 1*b*); proboscis and hind flagellum (Fig. 1*c*). The spherical object above this cell is another spermatozoid in the unexpanded state immediately after liberation from the antheridium. (Magnification × 3,000.)

Fig. 2. Another cell in side view, from material preserved in formalin and sea-water after killing with osmic vapour, showing proboscis and places of emergence of both flagella; the rest as in Fig. 1. (× 3,000.)

Fig. 3. Three other cells in various views, from the same preparation as Fig. 2, the rest as in Fig. 1. (× 3,000.)

Fig. 4. Electron micrograph M5·5 of a cell with an unusual development of a whiplash terminal extremity to the front flagellum, otherwise the body and 'Flimmer' in very good condition; for comparison with Figs. 1–3. (× 8,000.)

Fig. 5. Low-power electron micrograph M7·3, for comparison with Fig. 1. This is the same cell as Figs. 6, 8, and 9. (× 3,000.)

Fig. 6. More enlarged view of front end of the cell of Fig. 5. Electron micrograph M7·8. (× 9,000.)

Fig. 7. Another cell, electron micrograph M7·10. (× 6,000.)

PLATE XXI

Fig. 8. Enlarged view of body and bases of appendages of the cell of Fig. 5. Electron micrograph M8·3, uranium shadowing, reversed print, 80 kV. (× 40,000.)

Fig. 9. Enlarged view of the middle part of the front flagellum of the cell of Fig. 8 (cf. Fig. 6). Electron micrograph M8·2, uranium shadowing, reversed print, 80 kV. (× 40,000.)

PLATE XXII

Fig. 10. View of body contents in a cell, seen from above. Electron micrograph M9·6 at 100 kV.; *b*, basal granule; *e*, eyespot; *n*, nucleus. (× 10,000.)

Fig. 11. Side view of another cell, otherwise as in Fig. 10. Electron micrograph M9·5. (× 6,000.)

Fig. 12. Another cell, seen from below, otherwise as in Fig. 10. Electron micrograph M9·5. In addition to the greater clarity of outline of the main inclusion this view shows the putative sensory strand *s*. (× 6,000.)

Fig. 13. A flattened and partially decomposed cell showing contents unusually clearly. Lettering as in Fig. 10. Electron micrograph M9·5 at 100 kV. (× 6,000.)

Fig. 14. An enlarged view of the front end of the body of Fig. 22 to show putative sensory strand more clearly. Electron micrograph M25·5 at 80 kV. (× 20,000.)

Fig. 15. A large cell to show the attachment of the proboscis more clearly; arrows indicate thickened regions which appear to be related to the proboscis. Electron micrograph M29·1 at 80 kV. (× 15,000.)

Fig. 16. Detail of the free end of the proboscis. Electron micrograph M35·1, uranium shadowing, direct print, 60 kV. (×40,000.)

Fig. 17. The proboscis of a similar cell on another preparation. Electron micrograph M35·2, uranium shadowing, direct print, 60 kV. (×40,000.)

PLATE XXIII

Fig. 18. Two cells showing fibrillar disintegration of front flagellum; traces of 'Flimmer' and a small amount of other material visible. Approximately 11 strands in each. Electron micrograph M10·2, uranium shadowing, reversed print, 40 kV. (×20,000.)

PLATE XXIV

Fig. 19. Part of a cell showing fibrillar disintegration of both flagella, the front one pointing to the left, the back one pointing right. Electron micrograph M9·3 at 100 kV., uranium shadowing. (×20,000.)

Fig. 20. The same cell as Fig. 19, showing part of back flagella immediately beyond that visible in Fig. 19. Eight or nine component strands are visible on the right-hand portion. Electron micrographs M11·8 and 9 at 100 kV., uranium shadowing. (×20,000.)

Fig. 21. Tip of front flagellum of cell of Figs. 22 and 14 showing fibrillar disintegration beginning. Electron micrograph M25·6 at 80 kV. (×20,000.)

Fig. 22. Whole cell, showing the partially disintegrated front flagellum of Fig. 21. Electron micrograph M25·4 at 80 kV. (×10,000.) Part of the contents of this cell is reproduced at a higher magnification in Fig. 14.

I. MANTON and B. CLARKE


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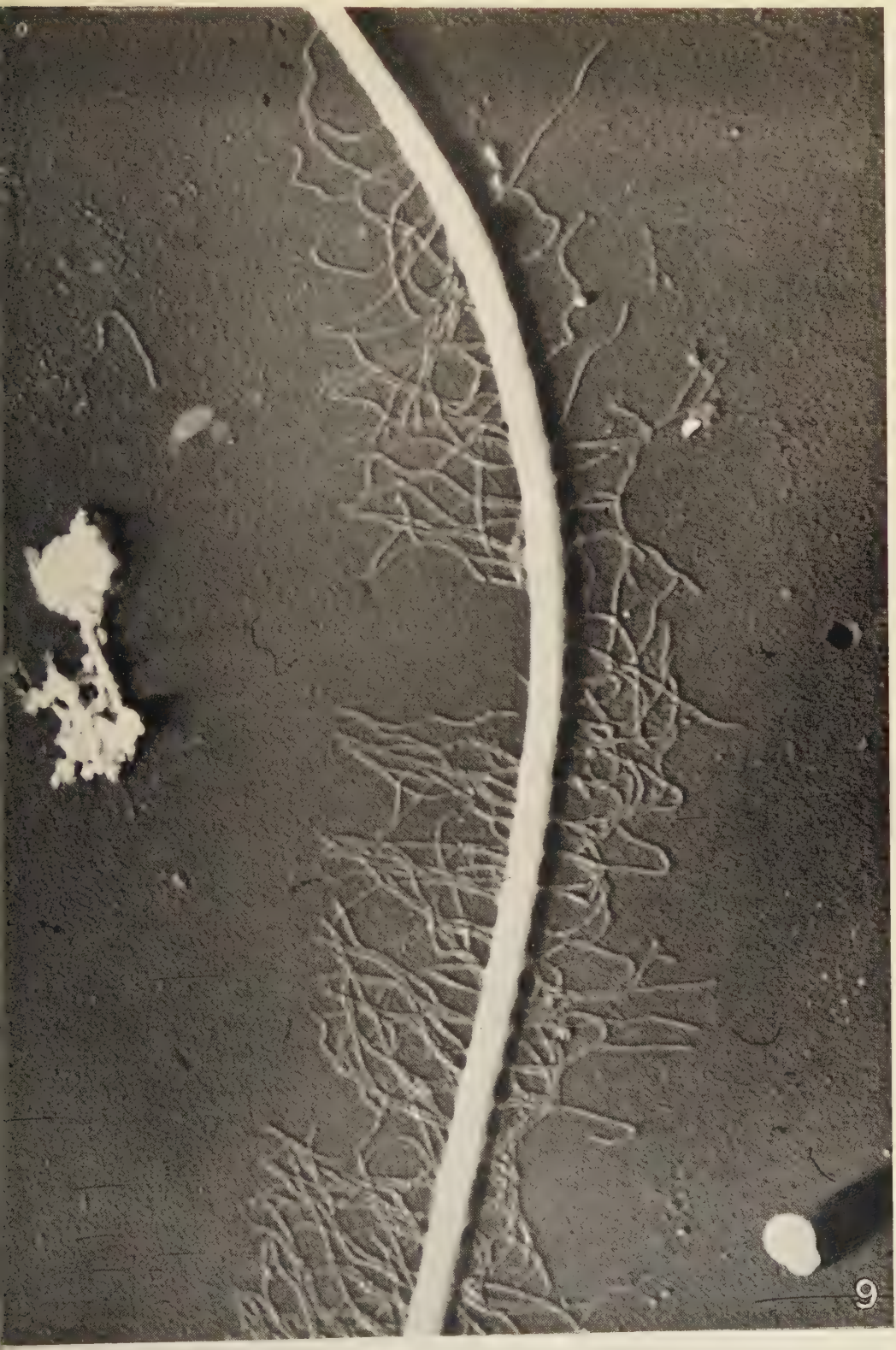
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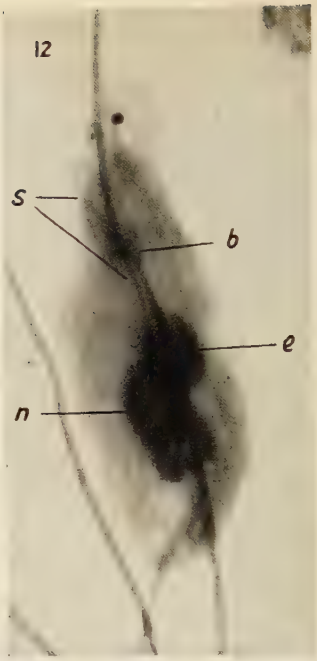
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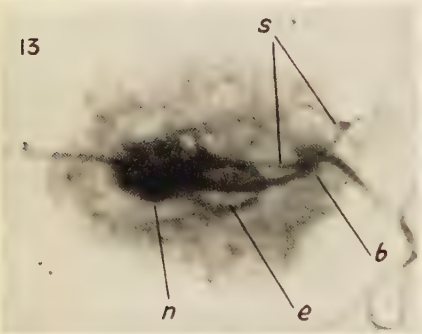
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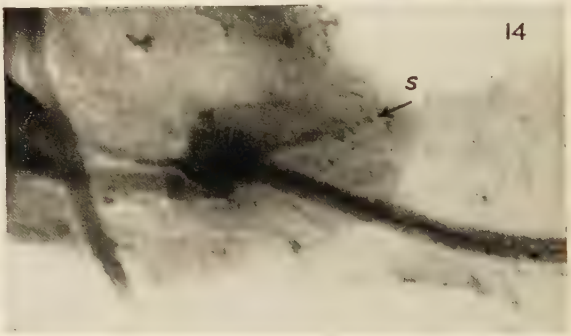
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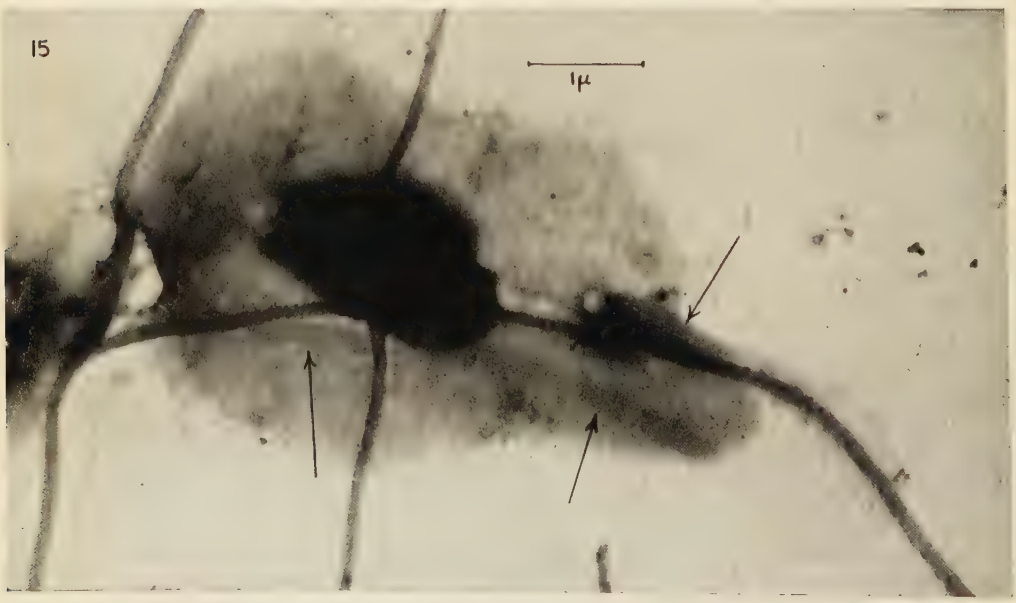
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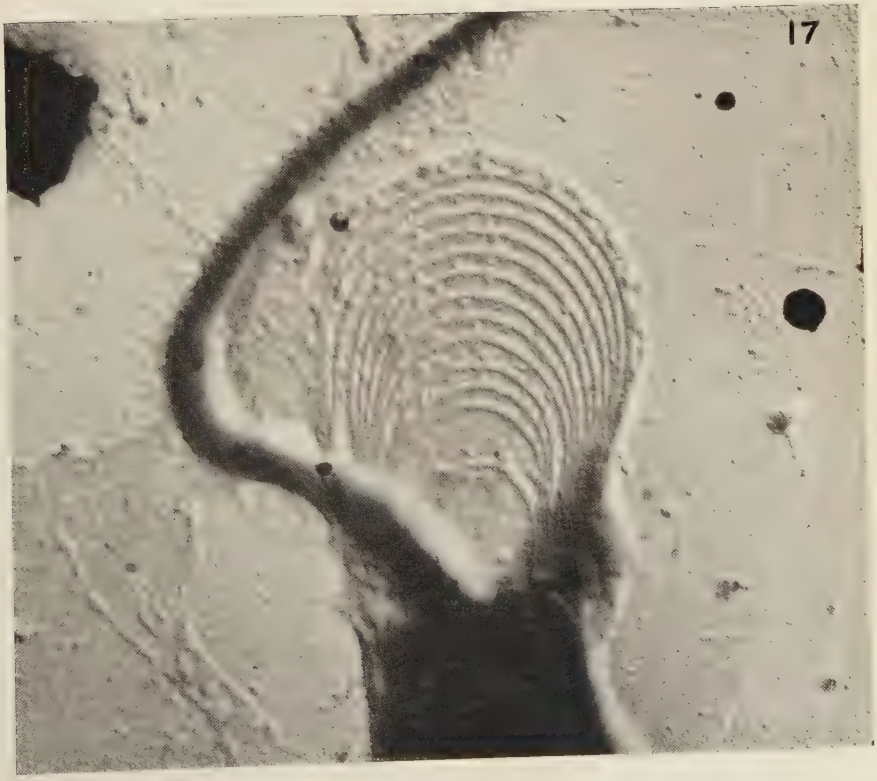
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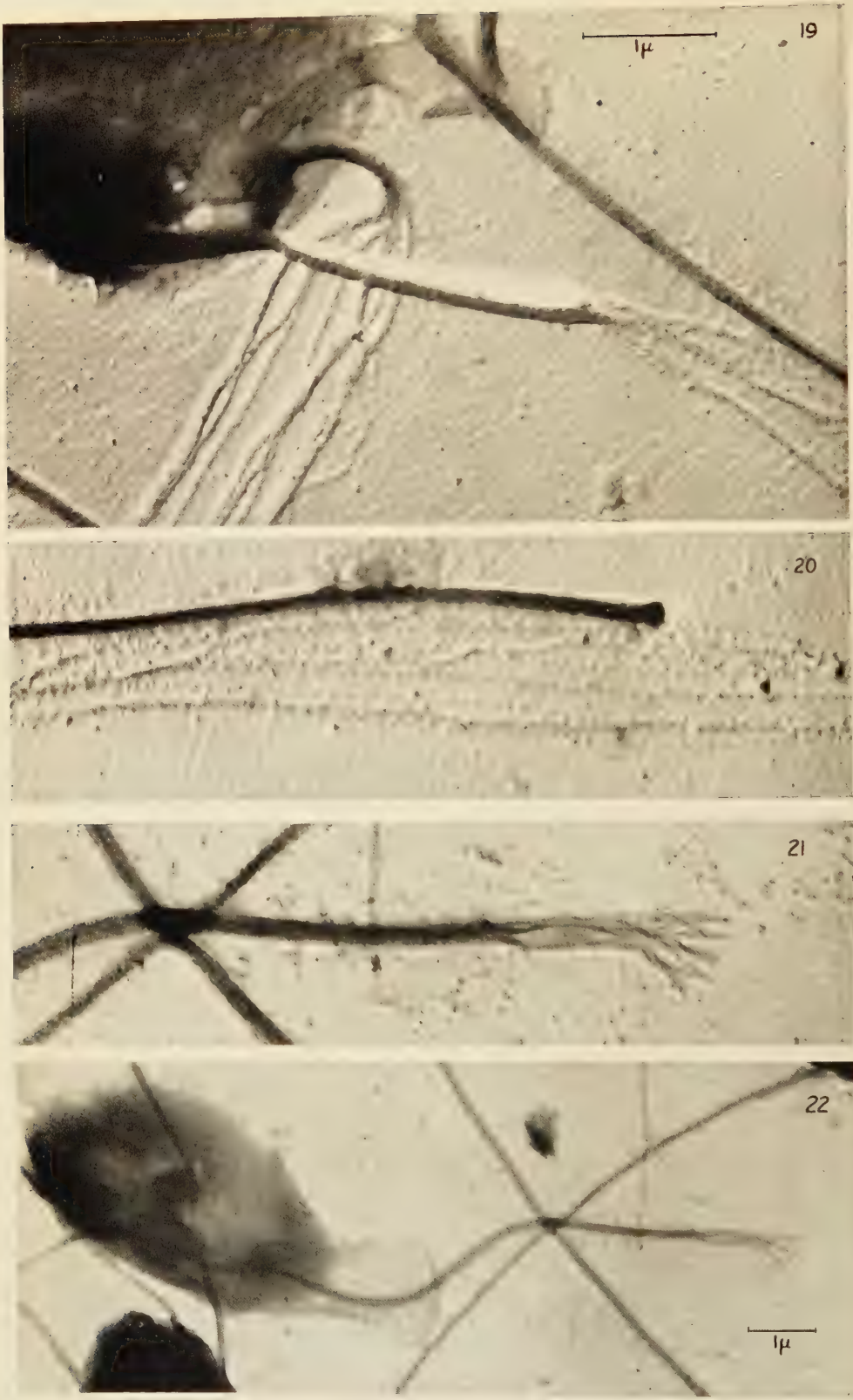
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The Effect of Cyanide on the Respiration and the Oxidative Assimilation of Glucose by *Chlorella vulgaris*

BY

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With six Figures in the Text

Abstract

It is shown that low concentrations of cyanide stimulate the endogenous respiration of *Chlorella vulgaris*. When glucose is added the respiration rate is much increased but is now reversibly inhibited by cyanide. Some 30–60 per cent. of the total respiration remains uninhibited. One-eighth to one-ninth of the glucose added is completely oxidized. Most of the remainder is assimilated to di- or polysaccharide. Low concentrations of cyanide which inhibit the rate of glucose oxidation also inhibit the assimilation of glucose. Two possible interpretations of this fact are discussed. It is suggested that the assimilation of glucose is coupled with the oxidation of glucose by a cyanide-sensitive respiratory system. The mathematical consequences of this theory are considered and shown to agree with the experimental results. The effect of cyanide on the respiratory quotient is also discussed.

Introduction

CLIFTON (1946) has reviewed much work which shows that when a source of carbon such as glucose is supplied to micro-organisms the substance is metabolized in such a way that part of it is oxidized to carbon dioxide and part assimilated as other carbon compounds. Assimilation in this sense is associated with the uptake of oxygen and is thus oxidative assimilation.

The oxidative assimilation of glucose by green algae has been studied by two workers. Myers (1947), using manometric methods to measure the gas exchanges involved, showed that when small quantities of glucose are added to *Chlorella pyrenoidosa* one-sixth of the added glucose is oxidized. The remaining five-sixths appears to be assimilated to some product similar to a carbohydrate in composition. He represented this by the equation

$$C_6H_{12}O_6+O_2 \longrightarrow 5(CH_2O)+CO_2+H_2O.$$

Taylor (1950) has studied the assimilation of glucose by *Scenedesmus quadricauda*. He also found that one-sixth of the added glucose was oxidized and he showed, by cell analyses, that most of the remainder was assimilated as polysaccharide.

The effect of poisons, such as dinitrophenol, sodium azide, and cyanide, on oxidative assimilation has also received some attention (Clifton, 1946).

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In general, the effect of adding such poisons to the organism is to decrease the proportion of the added carbon compound which is assimilated.

This paper deals with the effect of cyanide on the respiration of *Chlorella vulgaris* with particular reference to the effect of this poison on the oxidative assimilation of glucose.

Material and Methods

The alga used was a strain of *Chlorella vulgaris*. The growth and metabolism of this strain have been studied by Pearsall and Loose (1937). A pure culture of the alga was used and it was grown with the usual precautions against contamination.

The medium used for culture was similar to that used by Pearsall and Loose. It was prepared by mixing equal quantities of two solutions A and B. Solution A contained to each litre of water 0·4 g. of anhydrous magnesium sulphate, 1·5 g. of ammonium nitrate, 0·4 g. of sodium citrate, three drops of a saturated solution of ferrous sulphate, and 1·0 ml. of the A4 trace-element solution of Arnon (1938). Solution B contained 14·528 g. of potassium dihydrogen phosphate and 4·646 g. of dipotassium hydrogen phosphate per litre. This buffered the medium. Its pH after autoclaving was 6·0–6·1. The water used for preparing the solutions was 'Pyrex' distilled water. All the chemicals used, except the dipotassium hydrogen phosphate, were of ANALAR quality.

The cultures were grown in resistance-glass wash-bottles fitted with sintered glass discs. The total capacity of each wash-bottle was about 200 ml. 150 ml. of medium was put into each wash-bottle and autoclaved. After autoclaving the volume of medium in each bottle was about 145 ml.

Inoculation and growth of cultures

Sufficient inoculum from a liquid culture of known density was pipetted into each wash-bottle so that there were 100 cells per cu. mm. in each culture initially. The liquid cultures used for inoculation were prepared by transferring cells from an agar slope to 25 ml. of sterile medium in a 100-ml. conical flask. Such cultures were illuminated for 3 days and shaken occasionally.

After inoculation the wash-bottles were immersed in a water-bath maintained at 25° C. They were illuminated by a tungsten-filament lamp. The light intensity at the cultures was 600 foot-candles. The cultures were aerated with a sterile stream of air containing 0·5 per cent. carbon dioxide. The air was previously warmed and saturated with water by passing it through two wash-bottles containing sterile distilled water. Under these conditions the cells multiplied rapidly. After 48 hours each culture contained about 8,000 cells per cu. mm.

Unless otherwise stated the cells used for all the experiments described here were grown for 56 hours in the light. They were then darkened and

aerated for a further 16 hours. This depleted the cells of some of their carbohydrate reserves.

Measurement of respiration rate

The cultures were harvested by centrifuging at 1,500 r.p.m. The cells were then washed once and resuspended in fresh medium. Unless otherwise stated the respiration experiments were carried out with the cells suspended in the same medium as was used for their growth.

Gas exchange measurements were made by the Warburg manometric technique. All respiration experiments were carried out in a bath maintained at 25° C. The bath was darkened by black plastic material. When oxygen uptake was measured the centre-well of the Warburg flask contained 0·5 M caustic potash or a caustic potash-potassium cyanide mixture if cyanide was present. These centre-well solutions were those described by Robbie (1946).

Determination of carbohydrates

Methods similar to those described by Pearsall and Loose (1937) were used for the carbohydrate analyses of the cells. The cells from a Warburg flask were washed into a 15-ml. centrifuge tube. After centrifuging, the cells were washed once. The external medium and the washings were taken for analysis. The cells were killed by heating them quickly to 100° C. 5 ml. of 70 per cent. alcohol was then added and the cells extracted with this at 25° C. for 24 hours. A further extraction with 70 per cent. alcohol for 72 hours was made and then the combined extracts were made up to 25 ml. 5 ml. of this solution was taken for each determination of disaccharides (after hydrolysis at 100° C. for 10 minutes with 0·4 N sulphuric acid).

The total hydrolysable polysaccharide remaining in the cells was determined by boiling the cell debris in each centrifuge tube for 3 hours with 2 ml. of 3 per cent. sulphuric acid under a reflex condenser. After neutralization of the acid, the cell remains were extracted with hot water. The extract was made up to 25 ml. and 2 ml. taken for each determination of reducing value.

The method used for determining reducing value was that of Hagedorn and Jensen (1923) as modified by Fujita and Iwatake (1931).

Replicate determinations of the same extract agreed to 1 per cent.

The Effect of Cyanide on the Rate of Respiration

The effect of low concentrations of cyanide on the respiration of *Chlorella* has been investigated by Emerson (1927) and Genevois (1927, 1929). They found that low cyanide concentrations increased the rate of endogenous respiration (i.e. the rate when no carbon compounds were added). If glucose was added to the cells, however, a much higher rate of respiration followed and this was inhibited by low concentrations of cyanide.

These results have been confirmed. Fig. 1 shows the result of a typical experiment to determine the rate of respiration of the cells in the presence

of different cyanide concentrations. After 100 minutes glucose was added from the side-arm and the new rate of respiration determined.

It is clear that cyanide markedly inhibits the rate of exogenous respiration (i.e. the rate when an added carbon source is present). It is also clear that a definite cyanide-stable respiratory system exists which is only inhibited by much higher cyanide concentrations. The rate of oxygen uptake through the cyanide-stable system may be 30–60 per cent. of the total rate in the absence of cyanide.

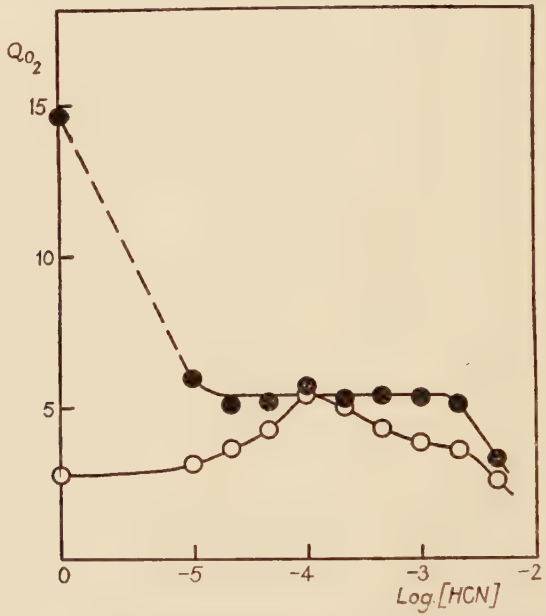

Fig. 1. The effect of cyanide on the rate of respiration.
-○—○- Endogenous respiration—no added substrate.
-●—●- In the presence of M/25 glucose.
The cells used were grown for 72 hours and illuminated the whole time.

The Reversibility of Cyanide Inhibition

The inhibition of glucose oxidation by cyanide can be completely reversed if the cyanide is removed by centrifuging and washing the cells. The results of such an experiment are given below. M/25 glucose was present in the suspending medium.

	Q_{O_2}
Control	14·7
10^{-4} M cyanide present . . .	8·8
After removing cyanide by washing and resuspending cells in fresh medium .	14·6

The inhibition is presumably due to the combination of cyanide with an enzyme system containing a heavy-metal. This is probably a cytochrome system since Emerson (1927) found that the carbon monoxide inhibition of the rate of glucose oxidation by chlorotic *Chlorella* was light reversible.

The Stimulation of Endogenous Respiration by Cyanide

It is noticeable that although the rate of endogenous respiration is stimulated by low cyanide concentrations the highest rate reached is never higher than the rate in the presence of glucose at the same cyanide concentration. This suggests that the rate of both endogenous and exogenous respiration in the presence of cyanide may be limited by the rate of the same respiratory system.

Warburg and Uyesugi (1924) have suggested that the stimulation of the rate of oxygen uptake of *Chlorella* by cyanide may be due to the oxidation of the cyanide added. This, however, cannot be the explanation here as the extra volume of oxygen taken up is much too large to be accounted for by the complete oxidation of the small quantities of cyanide added.

It has also been suggested (Blum, 1935) that the stimulation of oxygen uptake by cyanide is due to the inhibition of catalase. The hydrogen peroxide supposed to be produced during respiration is then not decomposed to oxygen and water. Hence apparently more oxygen is taken up during respiration. This, again, cannot be the complete explanation here as cyanide stimulates the rate of carbon dioxide production as well as the rate of oxygen uptake (Table I).

Table I

The Effect of Cyanide on the Oxygen Uptake and Carbon Dioxide Output of Chlorella

No added substrate. pH of medium = 5·2

CN conc. *M*	Q_{O_2}	Q_{CO_2}
0	1·71	2·08
$0{\cdot}22 \times 10^{-5}$	1·80	2·10
$0{\cdot}46 \times 10^{-5}$	2·08	2·38
$1{\cdot}0 \times 10^{-5}$	2·53	2·66
$0{\cdot}22 \times 10^{-4}$	4·13	4·13

The stimulation of endogenous respiration cannot be immediately reversed by centrifuging and washing the cells. The stimulation, then, is not simply due to the reversible combination of cyanide with some system inside the cells. Hanes and Barker (1931) have suggested that cyanide stimulates the respiration of potato tubers by increasing the concentration of sugar within the cells. No such increase in sugar concentration was found in *Chlorella* when treated with cyanide but only a very small increase may be required to increase the respiration rate appreciably. It is possible that cyanide stimulates the rate of respiration of the cells by increasing the availability of some subtrate which may or may not be sugar.

The Partial Oxidation of Glucose

When a small quantity of glucose is added to a suspension of partially starved *Chlorella* cells the rate of oxygen uptake increases markedly. The

rate remains high for some time but eventually falls to its original value (see Fig. 2). The respiratory quotient during the rapid oxidation of glucose is a little greater than unity (Table III).

Now if it is assumed that the oxidation of glucose is represented by the equation

$$C_6H_{12}O_6+6O_2 \longrightarrow 6CO_2+6H_2O,$$

the volume of oxygen required for the *complete* oxidation of the quantity of glucose added can be calculated. The volume of oxygen actually absorbed can be fairly accurately estimated from the breaks in the graph of oxygen uptake against time. This can then be compared with the calculated value. However, a difficulty is met when estimating the volume of oxygen actually absorbed since one has to decide whether the endogenous respiration continued during the oxidation of glucose or not.

The Estimation of the Volume of Oxygen Absorbed

Barker (1936) has presented evidence suggesting that the endogenous respiration of *Prototheca* is completely suppressed during the oxidation of glucose. Myers (1947) working with *Chlorella pyrenoidosa* and Taylor (1950) with *Scenedesmus quadricauda* have come to the same conclusion.

This is not an unreasonable assumption. Both endogenous and exogenous respiration may have some enzyme system in common, and this may be saturated in the presence of glucose. That such a common system exists is indicated by Fig. 1. If the two respiratory processes were completely independent the rate of oxygen uptake in the presence of glucose would be the sum of the rates for both systems. Then the curve of exogenous respiration would have a peak corresponding with that at 10^{-4} M cyanide concentration in the graph of endogenous respiration. Since this does not occur one can conclude that the two processes are linked in some way.

Further evidence that endogenous respiration is suppressed during the oxidation of glucose comes from the experiments in which cyanide was present and small quantities of glucose added. If it is assumed that endogenous respiration ceases during glucose oxidation, the results of experiments carried out at different times are in good agreement and there is a consistent increase in the volume of oxygen taken up as the cyanide concentration increases (see Fig. 3). If, on the other hand, it is assumed that endogenous respiration continues while the added glucose is being rapidly oxidized, the results of different experiments do not show the same consistencies.

One method of attempting to discover whether the endogenous respiration is suppressed or not has been frequently used. Different quantities of glucose are added to similar samples of cells and the volume of oxygen absorbed for each molecule of glucose added is calculated, assuming firstly that the endogenous respiration ceased during the oxidation of glucose and secondly that it continued. The assumption which gives the most consistent values for the volume of oxygen absorbed per micromole of added glucose is taken to

be the correct one. However, it can be shown that if the rate of endogenous respiration remains constant during the experiment and the transition from the rapid rate of glucose oxidation to the slower basic rate is sharp, both assumptions should give constant values of the volume absorbed. Any deviations will not be due to the falsity of the assumption but to errors either in the experimental results or in the determination of the breaks in the curves.

A slightly different approach has been used in this work. This involves a similar principle but is thought to be less open to criticism. The same quantity of glucose was added to cells from different cultures on different occasions. When this was done it was found that the ratio of the rates of endogenous to exogenous respiration varied slightly with different cultures. The volume of oxygen absorbed during the metabolism of the glucose was calculated assuming either that endogenous respiration continued or that it ceased. Two experiments may agree when one assumption is taken as correct, but then they will not agree when the other is used. Hence the assumption which gives the more constant values for the volume of oxygen absorbed is taken as more likely to be correct.

Ten experiments were made on six different occasions. In each 3 micromoles of glucose were added to the cells. The mean volume of oxygen taken up was:

	Mean.	Standard deviation.	Coefficient of variation.
Assuming endogenous respiration ceased	48·95	2·339	4·78
Assuming endogenous respiration continued	42·35	2·982	7·04

A comparison of the coefficients of variation shows that the results calculated taking the second assumption as correct are slightly more variable.

Thus the evidence indicates that the endogenous respiration is suppressed during the oxidation of glucose. If the 3 micromoles of glucose added in the experiments mentioned above were completely oxidized to carbon dioxide and water, the volume of oxygen absorbed would be 403·2 cu. mm. The mean value which was actually absorbed was 49 cu. mm. Hence only one-eighth of the added glucose was completely respired. What happens to the rest must now be considered.

The Oxidative Assimilation of Glucose

The experiment described here was performed to discover what happens to that part of the added glucose which is not oxidized. Since only one-eighth of the added glucose can be accounted for as carbon dioxide the remainder must either be present in the cells in such a position that it cannot be oxidized or have been converted to something else. The possibility that oxidation ceases because some inhibitor is produced can be ruled out by adding more glucose when the oxidation of that originally added has ceased. It is then found that this second quantity of glucose is partially oxidized just

as was the first. Hence nothing is inhibiting glucose oxidation. The work of Myers (1947) suggests that most of the glucose added to a suspension of *Chlorella pyrenoidosa* is converted to other carbohydrates. Therefore, in this experiment, the disaccharide and polysaccharide content of the cells was determined before and after the metabolism of the added glucose.

Method

The cells used were from 4-day-old cultures which were darkened for 16 hours before they were harvested. The cells were then centrifuged,

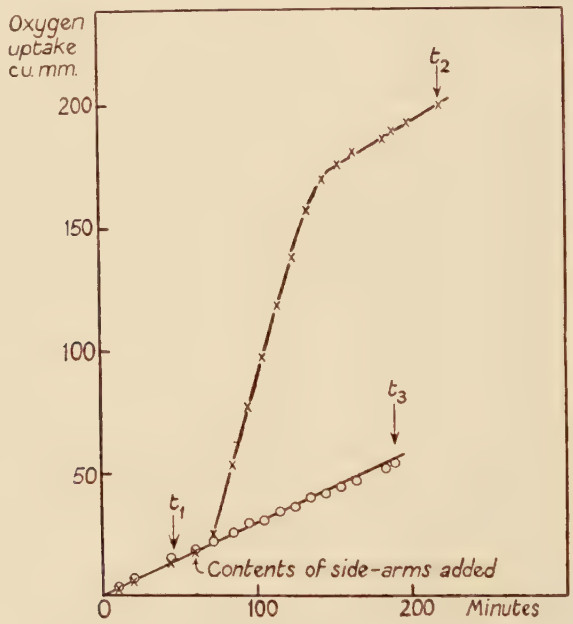

FIG. 2. The oxidative assimilation of glucose.

-X—X- 10 micromoles glucose added from side-arm after 60 mins.
-O—O- Control.

t_1, t_2, and t_3 are the times at which the various flasks were sampled (see text and Table II).

washed, and resuspended in culture medium as usual. 2 ml. of cell suspension was placed in each of nine Warburg flasks. The side-arms of five of these contained 10 micromoles glucose dissolved in culture medium, those of the others contained culture-medium only. The remaining cell suspension, contained in a 100-ml. conical flask, was shaken in the same tank as the Warburg flasks. All the flasks were in complete darkness at 25° C. After shaking for 45 minutes four 2-ml. samples of the stock suspension were taken for analysis. Soon after this the contents of the side-arms were added to the suspensions in the Warburg flasks. The oxygen uptake was followed, and when rapid oxidation ceased the cells from seven of the flasks were removed for analysis. The oxygen uptake of the two remaining flasks was followed for a further period.

The samples for analysis were centrifuged and the cells washed once. This took about 15 minutes. The reducing value of the suspending medium was determined. The cells were analysed for reducing sugar, disaccharide, and acid-hydrolysable polysaccharide.

Results

The oxygen uptake of the cells in the two flasks which were not sampled is plotted in Fig. 2. The times at which cells from the other flasks were removed for analysis are shown. The oxygen uptake in replicate flasks did not differ by more than 5 per cent. The results of the analyses are summarized in Table II.

TABLE II

The Oxidative Assimilation of Glucose by Chlorella

10 micromoles, i.e. 1,800 μ g. glucose were added to 2-ml. cell suspension. All analyses are given as μ g. equivalent glucose per 2-ml. cell suspension. t_1, t_2, t_3 refer to the sampling times which are shown in Fig. 2.

	Original suspension sampled at t_1 (1)	Suspension to which glucose was added sampled at t_2 (2)	Suspension to which no glucose was added sampled at t_3 (3)	Increase after addition of glucose (2)–(1)	Probability of such a difference occurring by chance
No. of replicates	4	4	3	—	—
Reducing value of external medium	20·8 ± 11·05	9·3 ± 5·87	5·67 ± 6·99	−11·5 ± 12·51	0·3–0·4
Reducing value of cell abstract	323·0 ± 5·05	340·0 ± 8·69	320·3 ± 10·14	17·0 ± 10·05	0·1–0·2
Disaccharides in cell extract	127·8 ± 16·08	445·8 ± 2·49	128·0 ± 8·78	318·0 ± 16·28	< 0·001†
Total insoluble carbohydrate in the cells	1,958·8 ± 16·15	2,900·0 ± 7·36	1,865·3 ± 7·43	941·2 ± 17·75	< 0·001†
Amount respired*	—	206·3 ± 1·31	51·0 ± 1·15	206·3 ± 1·31	—
			TOTAL	1,471·0 ± 28·97.	

∴ The analysis accounts for 81·7 ± 1·61% of the added glucose.

* Calculated from the volume of oxygen taken up. † This difference is highly significant.

Discussion

No change in the reducing value of either the medium or the cell extract was found to have occurred when the samples were analysed. It is clear, then, that none of the added glucose remains, as such, in either the medium or the cells when the rapid rate of oxygen uptake ceases. In this experiment the volume of oxygen absorbed corresponded to the complete oxidation of a little over one-ninth of the added glucose. The results show that there was a significant increase of both the disaccharide and polysaccharide fractions of the cells. Taken together the increase of these substances accounts for rather more than two-thirds of the added glucose. In all just over 80 per cent. of the glucose added can be accounted for. The rest must have been converted to other substances, possibly protein. One can conclude, therefore, that when small quantities of glucose are added to *Chlorella* cells some of the glucose is completely oxidized but by far the greater part is synthesized

to more complex substances, chiefly polysaccharide. Thus *Chlorella*, like other organisms (Clifton 1946), oxidatively assimilates glucose.

It is interesting to compare these results with those of other workers using related organisms. Myers (1947) showed that *Chlorella pyrenoidosa* completely oxidized one-sixth of the added glucose. The respiratory quotient, as in these experiments with *Chlorella vulgaris*, was just over one. Myers suggested that the remainder of the glucose was synthesized to substances of carbohydrate nature. Taylor (1950) found that one-sixth of the glucose added to *Scenedesmus quadricauda* was completely oxidized. He analysed the cells after oxidation had ceased and found that most of the glucose was converted to insoluble carbohydrate. He was able to account for 84 per cent. of the added glucose. Thus these organisms and the strain of *Chlorella vulgaris* studied here behave similarly except that *Chlorella vulgaris* oxidizes completely a smaller proportion of the glucose added.

The Effect of Cyanide on the Oxidative Assimilation of Glucose

When small quantities of glucose are added to *Chlorella* cells in the presence of low cyanide concentrations, the volume of oxygen taken up during metabolism of the glucose is greater than that taken up when no cyanide is present. At the same time, cyanide decreases the *rate* at which the glucose is oxidized by the cells.

Four experiments were made; for each a range of cyanide concentrations was used and 3 micromoles glucose added to the cells. The results are expressed in Fig. 3. Both the rate of glucose oxidation, expressed as a percentage of the control rate, and the volume of oxygen taken up when 3 micromoles glucose are metabolized, are plotted against cyanide concentration. It can be seen that the rate of glucose oxidation decreases with increasing cyanide concentration. But it is also clear that as the cyanide concentration is increased over the same range the volume of oxygen taken up increases.

This fact is more obvious when the results are plotted as in Fig. 4. Here the volume of oxygen taken up during the metabolism of 3 micromoles glucose is plotted against the rate of oxygen uptake (expressed as a percentage of the rate when no cyanide is present). It is clear that as the one increases, the other decreases. A curve can be drawn through the points and there is no evidence that the volume of oxygen taken up is increased by cyanide concentrations which do not decrease the *rate* of oxygen uptake.

The Effect of Cyanide on the Respiratory Quotient

Two experiments were carried out to determine the effect of cyanide on the respiratory quotient of *Chlorella* before, during, and after the assimilation of glucose. The direct method of Warburg was used (Dixon, 1943). The cells were suspended in phosphate buffer. The pH of this was accurately determined and correction made for carbon dioxide retention by the method

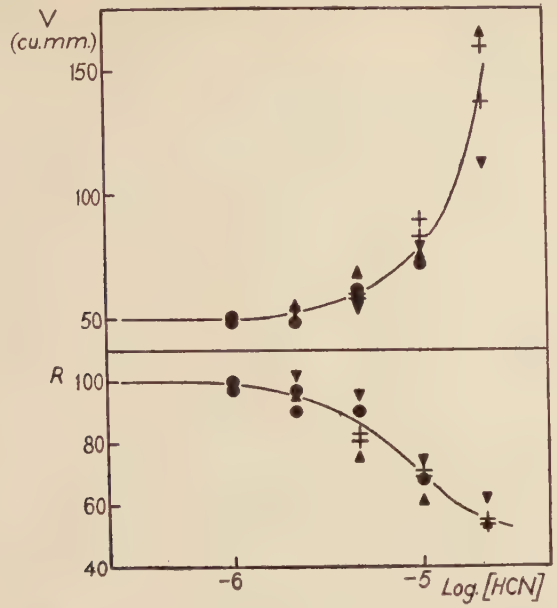

FIG. 3. During each experiment 3 micromoles glucose were added to the cells from the side-arm of a Warburg flask. The upper part of the figure shows the volume of oxygen (V) absorbed during the metabolism of the glucose. The lower part shows the rate of oxidation of the glucose (R) in the same experiments plotted as a percentage of the rate when no cyanide is present. Both V and R are plotted against the logarithm of the cyanide concentration. The different symbols refer to four different experiments which were carried out on different occasions. In each a range of cyanide concentrations was used. The values of V when no cyanide is present are omitted for clarity. There were six values all between 47 and 51 cu. mm.

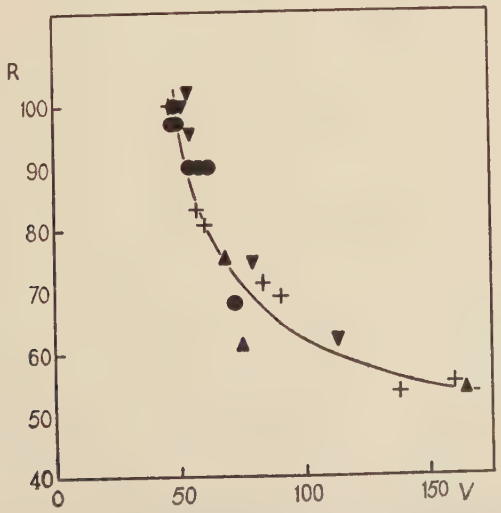

FIG. 4. The results of the experiments shown in Fig. 3 plotted to show the relationship between V and R. Not all the values of V when $R = 100$ are shown as they are so close together. (For further explanation see text and legend to Fig. 3.)

of Johnson (see Umbreit, Burris, and Stauffer, 1949). The results are shown in Table III.

TABLE III

The Effect of Cyanide on the Respiratory Quotient of Chlorella

HCN conc.	Before adding glucose		In presence of glucose		After oxidation of glucose has ceased	
	XLI	XLII	XLI	XLII	XLI	XLII
0	1·22	1·34	1·07	1·05	1·14	1·42
$0{\cdot}22\times10^{-5}$	1·17	1·29	1·01	1·03	1·13	1·42
$0{\cdot}46\times10^{-5}$	1·14	1·18	1·04	1·01	1·07	1·28
$1{\cdot}0\times10^{-5}$	1·05	1·22	1·24	1·11	1·05	1·09
$0{\cdot}22\times10^{-4}$	1·00	1·00	1·12	1·12	(1·07)*	1·02

* This figure is not reliable.

These figures show that the respiratory quotient of endogenous respiration is normally 1·2–1·4. As the cyanide concentration is increased the value becomes closer to unity. The respiratory quotient of exogenous respiration, on the other hand, is only a little greater than unity. It appears to be slightly larger in the presence of cyanide.

DISCUSSION

The effect of cyanide on the oxidative assimilation of glucose

The results presented here show that some of the glucose added to a *Chlorella* suspension is completely oxidized to carbon dioxide but by far the greater part of it is synthesized to more complex substances. In other words, synthesis is coupled with oxidation. It is possible that the energy required for synthesis is obtained from the accompanying oxidation. In the presence of low cyanide concentrations more of the added glucose is respired and less synthesized to the more complex substances. The same cyanide concentrations also inhibit the rate of glucose oxidation (Fig. 3).

There are at least two possible interpretations of these results.

(*a*) That cyanide has two effects:

(i) The inhibition of some heavy-metal enzyme system concerned with part of the total respiration. This is how cyanide is generally supposed to act.

(ii) The interference with the conversion of glucose to more complex substances. Dinitrophenol (Loomis and Lipmann, 1948) and sodium azide (Spiegelman, Kamen, and Sussman, 1948) appear to interfere with assimilation in this way. These substances appear to stop the coupling of synthesis with oxidation by interfering with phosphorylation.

(*b*) That cyanide has one effect only, namely, that usually assigned to it—the inhibition of one of the heavy-metal enzyme systems concerned with

respiration. The work of Emerson (1927) suggests that this is a cytochrome system. *Chlorella* also possesses a cyanide-stable respiratory system which can oxidize glucose at an appreciable rate (Fig. 1). If, however, synthesis can only be coupled with glucose oxidation which takes place through the cyanide-sensitive system, the results would be explained. Thus at low cyanide concentrations which partially inhibit the cyanide-sensitive system a smaller proportion of the glucose oxidation would pass through this system and hence less synthesis would occur. This interpretation is the simpler in that low

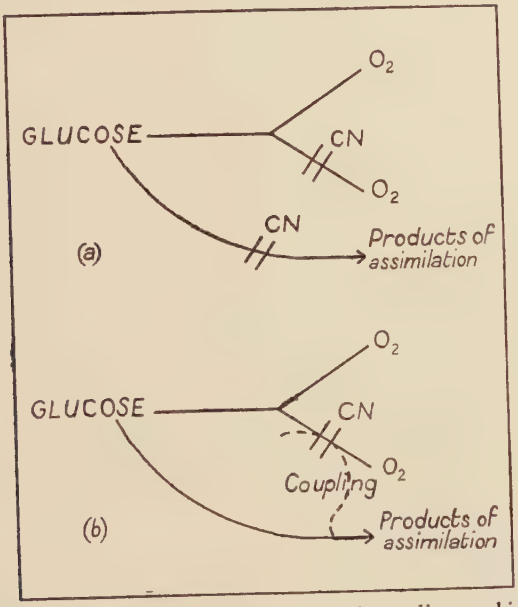

FIG. 5. Diagrammatic representation of the two hypotheses discussed in the text: (*a*) assuming cyanide inhibits two processes; (*b*) assuming cyanide inhibits one process with which assimilation is coupled.

cyanide concentrations are assumed to inhibit only one enzyme system within the cell.

These two alternative interpretations are represented diagrammatically in Fig. 5.

If the first interpretation was correct and cyanide has two independent effects on the cells' metabolism, there is no reason why both the rate of oxidation and assimilation should be inhibited by the same concentration of cyanide. Low concentrations of dinitrophenol inhibit the oxidative assimilation of glucose by *Escherichia coli* (Clifton, 1946), but they do not inhibit the rate of oxygen uptake. A low concentration of sodium azide prevents assimilation of glucose by fermenting yeast cells, but it does not inhibit the rate of fermentation (Spiegelman et al., 1948). Evidently the second interpretation proposed above cannot be correct for these substances.

There is evidence, however, that cyanide acts differently. It can be seen

that low cyanide concentrations inhibit both the assimilation and the oxidation of glucose by *Chlorella*. A cyanide concentration which depresses the one depresses the other. This suggests that cyanide inhibits something common to both processes. Moog and Spiegelman (1942) have shown that sodium azide markedly inhibits the regeneration of *Tubularia* at concentrations which have little effect on the rate of respiration. Cyanide also inhibits regeneration, but only at concentrations which greatly inhibit the rate of respiration. Thus here cyanide and azide differ in their effects. In a later paper Spiegelman and Moog (1945) question the assumption which is sometimes made that azide and cyanide are equivalent inhibitors of cell metabolism. Winzler (1940) found that low concentrations of dinitrophenol did not depress the rate of oxidation of acetate by yeast but prevented its assimilation. He found that cyanide also prevented the assimilation of acetate, but his results show that when this happened the rate of acetate oxidation was decreased. It seems worthwhile, therefore, to examine this second possibility further.

If certain assumptions are made the second hypothesis can be expressed mathematically. It can then be seen whether the experimental results agree with the mathematical consequences of this hypothesis.

To derive the mathematical expression it is assumed that the total exogenous respiration can be separated into two fractions, one cyanide-stable and the other cyanide-sensitive. The assimilation of glucose is assumed to be coupled with the oxidation of glucose by the cyanide-sensitive system in such a way that for every molecule which is oxidized by this path a certain fixed number of molecules are synthesized to some more complex substances. This number is assumed to be independent of the cyanide concentration. The inhibition of the cyanide-sensitive respiratory system is assumed to be the only effect of cyanide.

If these assumptions are made an expression can be derived which connects the volume of oxygen taken up (V) during the metabolism of a given quantity of glucose with the rate of respiration (R) expressed as a percentage of the control rate in the absence of cyanide. The full derivation of this expression is given in the Appendix. It is:

$$V = \frac{K_1 R}{K_2 R - K_3}.$$

K_1, K_2, and K_3 are constants which are functions of:

(*a*) The volume of oxygen taken up during the metabolism of a given quantity of glucose in the absence of cyanide. This value is obtained from the experimental results. In these experiments the mean value was 49·0 cu. mm. for 3 micromoles of added glucose.

(*b*) The volume of oxygen which would have been taken up had all the glucose added been completely oxidized according to the equation

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O.$$

This, for 3 micromoles glucose $= 22{\cdot}4 \times 6 \times 3 = 403{\cdot}2$ cu. mm.

(*c*) The rate of the cyanide-stable respiration expressed as a percentage of the total rate of respiration in the absence of cyanide.

This, too, should be obtainable from the experimental results. In these experiments, however, the cyanide concentrations used were not high enough

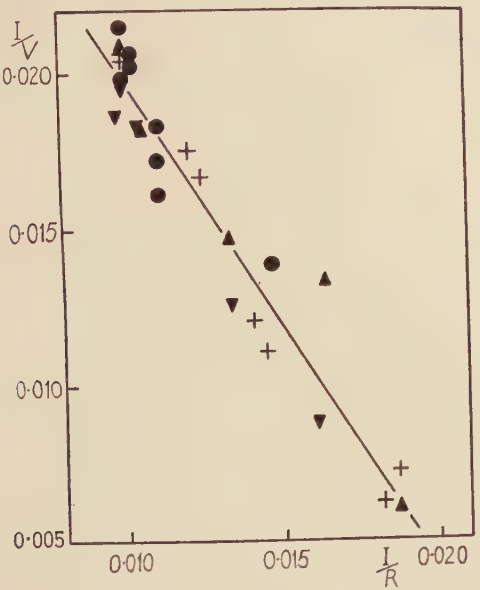

Fig. 6. (For explanation see text.)

to inhibit completely the cyanide-sensitive respiratory system (Fig. 3). A comparison of Figs. 1 and 3 suggests that the cyanide-stable respiration was probably about 50 per cent. of the total.

It is now possible to compare the experimental results with the mathematical expression which has been derived. It is easier to do this if the expression is written as:

$$\frac{1}{V} = \frac{K_2}{K_1} - \frac{K_3}{K_1} \cdot \frac{1}{R}.$$

Thus, if this interpretation of the results is correct, when the reciprocal of the volume of oxygen absorbed is plotted against the reciprocal of the respiration rate the points should lie on a straight line. The results are shown plotted in this way in Fig. 6. The points do, in fact, fall approximately on a straight line. The correlation coefficient is −0·96. The results, therefore, agree with this hypothesis in that respect.

A line[1] which fits the points has been drawn. The equation of this line is

$$\frac{1}{V} = 0{\cdot}03566 - \frac{1{\cdot}577}{R}.$$

[1] The equation of this line was calculated by Mr. N. W. Please. It was obtained by minimizing the sum of squares of the perpendicular distances of the points from the line. This

The equation theoretically deduced agrees with this numerically if the cyanide-stable respiration is assumed to be 47 per cent. of the total respiration. As suggested above this is not an unlikely assumption. It is impossible to decide from these results exactly what percentage of the total respiration is cyanide-stable. One can only say that it is probably about 50 per cent.

It is fair to conclude, then, that the second explanation proposed above can explain these results. When its mathematical consequences are examined they agree with what is found by experiment. Thus it would seem that the effect of cyanide on the rate of respiration and amount of assimilation of glucose by *Chlorella* can be explained by assuming that low cyanide concentrations inhibit one enzyme system only. Hence assimilation of glucose is thought to be coupled with the oxidation of glucose by this cyanide-sensitive system. Some glucose is also oxidized by the cyanide-stable system, but this oxidation is not coupled with assimilation.

Now in the absence of cyanide about 8–9 molecules of glucose are assimilated for each one that is completely oxidized. But if this idea is correct and oxidation of glucose through the cyanide-stable system is not coupled with assimilation it follows that the oxidation of one molecule by the cyanide-sensitive system must be coupled with the assimilation of 13–14 molecules of glucose (see Appendix). This figure is much greater than any that has been previously proposed for assimilation. It is, however, far from impossible on energetic grounds. The condensation of glucose to polysaccharide takes place with an increase of free energy of not more than 6 Cals. per glucose unit. Thus the condensation of 13–14 glucose units would occur with an increase of free energy of not more than 78–84 Cals. The complete oxidation of one glucose molecule, on the other hand, occurs with a decrease of free energy of about 700 Cals. Thus ample energy for the assimilation of 13–14 glucose molecules should be released by the complete oxidation of one of them.

The effect of cyanide on the respiratory quotient

The change in the value of the respiratory quotient with increasing cyanide concentration is interesting (Table III). When neither glucose nor cyanide is present the respiratory quotient is considerably greater than 1·0, but as the cyanide concentration is increased it becomes nearer unity. The results presented in Table II show that endogenous respiration takes place largely at the expense of acid-hydrolysable polysaccharide. One might, therefore, have expected the respiratory quotient to be 1·0 corresponding to carbohydrate oxidation. However, Cramer and Myers (1948) have shown that the respiratory quotient of *Chlorella pyrenoidosa* is altered when the cells are assimilating nitrogen. They found that the respiratory quotient of cells assimilating ammonia in the presence of glucose in the dark was 1·2. They showed that a respiratory quotient greater than 1·0 was to be expected if

was thought to give the best estimate of the functional relationship between the variables. This line lies between the two regression lines which can be fitted to the observations.

the cells utilized both carbohydrate and ammonia for growth. The actual value of the respiratory quotient depends on the relative amounts of carbohydrate which are respired and assimilated. The experiments described here were carried out with the cells suspended in a medium containing ammonium nitrate. Pearsall and Loose (1937) and Cramer and Myers (1948) have shown that *Chlorella* utilizes ammonia rather than nitrate from such a medium. Thus the cells in these experiments were growing at the expense of carbohydrate within the cells and ammonia supplied in the medium. Consequently a respiratory quotient greater than unity is not unexpected.

When cyanide is added the respiratory quotient of endogenous respiration falls to a value nearer unity. This strongly suggests that cyanide interferes with the assimilation of ammonia. For if this is inhibited the respiratory quotient would become nearer 1·0—the value for carbohydrate oxidation when uninfluenced by other factors. It is interesting to note that the cyanide concentrations which appear to inhibit ammonia assimilation are just those concentrations which inhibit the assimilation and rate of oxidation of glucose. It is hoped to consider the significance of this more fully in a later paper.

During the assimilation of glucose the respiratory quotient is only slightly greater than unity. The difference between this value and that of 1·2 found by Cramer and Myers (1948) is presumably due to the different treatment of the cells. The cells were starved for 16 hours before being used in these experiments. When glucose was added to them its oxidation and assimilation to more complex carbohydrates would appear to have been so rapid compared to the assimilation of glucose and ammonia for growth that the respiratory quotient is only slightly higher than unity. An increase of the cyanide concentration is followed by a slight rise of the respiratory quotient of glucose oxidation. This suggests that a little fermentation may occur at higher cyanide concentrations when glucose is present.

Appendix

Mathematical Formulation of the Hypothesis that Glucose Assimilation is coupled with Oxidation by the Cyanide-sensitive System only

It is assumed that glucose is respired by two enzyme systems only one of which is inhibited by cyanide. It is assumed that z molecules of glucose are assimilated for every one which is oxidized by this cyanide-sensitive system. The value of z is assumed to be independent of the cyanide concentration.

Let R = rate of respiration when cyanide is present expressed as a percentage of the rate in the absence of cyanide.

R_s = rate of cyanide-stable respiration expressed as a percentage of the rate in the absence of cyanide.

V = volume of oxygen taken up during the metabolism of the added glucose.

Let V_0 = volume of oxygen taken up during the metabolism of the same quantity of glucose in the absence of cyanide.

T = volume of oxygen which would have been taken up had all the added glucose been completely oxidized to carbon dioxide.

The mathematical treatment is simplified if quantities of glucose are represented by the volumes of oxygen required to oxidize them completely, i.e. 1 micromole glucose is equivalent to $6 \times 22{\cdot}4 = 134{\cdot}4$ cu. mm. oxygen. T then represents the quantity of glucose added to the cells.

Now the quantity of glucose completely oxidized

$$= V = aR \qquad \text{and} \qquad a = \frac{V}{R}, \tag{1}$$

where a is proportional to the time for which glucose oxidation continued.

The quantity of glucose respired through the cyanide-sensitive system

$$= a(R-R_s). \tag{2}$$

$\therefore$ The quantity of glucose assimilated

$$= az(R-R_s). \tag{3}$$

The total glucose metabolized = that respired + that assimilated.

$$\therefore \quad T = aR + az(R-R_s).$$

Now $a = V/R$ from (1).

$$\therefore \quad T = V + \frac{V}{R} z(R-R_s). \tag{4}$$

When no cyanide is present $V = V_0$ and $R = 100$.

$$\therefore \quad T = V_0 + \frac{V_0 z}{100} (100-R_s)$$

and

$$z = \frac{100(T-V_0)}{V_0(100-R_s)}. \tag{5}$$

From (4)

$$V = \frac{RT}{z(R-R_s)+R}.$$

Substituting the value of z given by (5)

$$V = \frac{RT}{\frac{100(T-V_0)(R-R_s)}{V_0(100-R_s)} + R}$$

$$= \frac{RTV_0(100-R_s)}{R(100T-R_sV_0)-100R_s(T-V_0)}$$

$$= \frac{K_1 R}{K_2 R - K_3}, \tag{6}$$

or
$$\frac{1}{V} = \frac{K_2}{K_1} - \frac{K_3}{K_1} \cdot \frac{1}{R}, \tag{7}$$

where
$$K_1 = TV_0(100 - R_s),$$
$$K_2 = 100T - R_sV_0,$$
$$K_3 = 100R_s(T - V_0).$$

Thus K_1, K_2, and K_3 are functions of T, V_0, and R_s. Now the equation of the line of best fit to the experimental points is

$$\frac{1}{V} = 0{\cdot}03566 - \frac{1{\cdot}577}{R} \quad \text{(see p. 487)}.$$

∴ If this hypothesis is to agree with the results

$$\frac{K_2}{K_1} = \frac{(100T - R_sV_0)}{TV_0(100 - R_s)} \quad \text{must equal } 0{\cdot}03566,$$

and

$$\frac{K_3}{K_1} = \frac{100R_s(T - V_0)}{TV_0(100 - R_s)} \quad \text{must equal } 1{\cdot}577.$$

In these experiments 3 micromoles glucose were added

$$V_0 = 49 \text{ cu. mm.} \quad \text{and} \quad T = 403{\cdot}2 \text{ cu. mm.}$$

It follows that if the mathematical formulation of this hypothesis is to agree exactly with the experimental results one must assume R_s to be 46·8 per cent.

Calculation of the value of z

If R_s is assumed to have this value it also follows that

$$z = \frac{100(T - V_0)}{V_0(100 - R_s)} \tag{5}$$
$$= \frac{100(403{\cdot}2 - 49)}{49(100 - 46{\cdot}8)}$$
$$= 13{\cdot}6,$$

i.e. 13·6 molecules of glucose are assimilated for each one which is oxidized completely by the cyanide-sensitive enzyme system.

I should like to thank Professor W. H. Pearsall for his helpful criticism of this paper and Mr. N. W. Please of the Statistics Department of University College, London, for his help with the statistics.

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Phytotoxic Effects of some Antibiotics

BY

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Abstract

The toxicity of a number of antibiotics, coumarin, and 3-indolylacetic acid to wheat, clover, and mustard seedlings has been assessed by a simple germination test. The percentage germination of wheat was not markedly affected by any of the compounds tested although root growth was reduced. Severe inhibition of root growth of the other seeds was usually accompanied by reduction in percentage germination.

None of the antibiotics tested were as inhibitory as indolylacetic acid, but most were more toxic than coumarin. The more toxic of the antibiotics were alternaric acid, glutinosin, mycophenolic acid, and gliotoxin. The least toxic were griseofulvin, penicillin, and streptomycin. Higher concentrations of the latter antibiotic were found to have an inhibiting effect on the synthesis of chlorophyll.

Introduction

A NUMBER of antibiotics have shown promise of being of practical value in plant pathology. For example, penicillin is effective in the treatment of crown gall (Brown and Boyle, 1944); actidione (Felber and Hamner, 1948; Gottlieb, Hassan, and Linn, 1950) and antimycin (Leben and Keitt, 1947, 1948, 1949) have been used as fungicidal foliage sprays; gliotoxin shows some activity when used as a fungicidal seed dressing (Brian and Hemming, 1945); griseofulvin has shown promise as a systemic fungicide (Brian, Wright, Stubbs, and Way, 1951). It has therefore been thought of value to assess roughly the phytotoxicity of antibiotics studied in these laboratories; for this purpose a simple germination test has been used.

Experimental Materials and Methods

The substances tested are listed in Tables I, II, and III; they include twelve natural antibiotics, one derivative of a natural antibiotic—griseofulvic acid—and two synthetic inhibitors, coumarin and 3-indolylacetic acid, included for purposes of comparison. All the antibiotics were prepared at these laboratories except penicillin (I.C. Pharmaceuticals Ltd.) and streptomycin (Boots Pure Drug Co. Ltd.). The streptomycin had an activity of 523 units/mg.; all other substances were substantially pure.

The seeds used throughout the experiment were wheat (vars. 'Drottnings White' and 'Holdfast'), white mustard, and red clover. They were sown in

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Petri dishes on agar containing the antibiotics at concentrations of 25, 5, 1, and 0 p.p.m.; this agar medium was made by mixing hot tap-water agar with a solution of the antibiotic or other inhibitor in McIlvaine's buffer at pH 4 or pH 6. Each plate contained 12 wheat seeds or 15 clover or mustard seeds and all treatments were replicated four times. The plates were stacked with the agar surfaces vertical and incubated at 25° C. for 4 or 5 days. The number of seeds germinated was then recorded and the amount of root formed was measured. The measure of root growth was fresh weight in the case of wheat, length of the root system in the case of the other two seeds.

Results

The results are presented in Tables I, II, and III. The data given are percentage germination and root growth expressed as percentage of that observed in the controls.

Table I

Toxicity of Various Antibiotics, 3-Indolylacetic Acid, and Coumarin to Wheat Seeds

Compound.		% germination				Root growth as % of control		
		25	5	1	0 p.p.m.	25	5	1 p.p.m.
3-Indolylacetic acid	a[1]	65	86	96	86	7	37	55
	b	50	81	90	73	6	28	49
Coumarin	a	89	98	100	100	20	65	107
	b	88	98	100	100	18	71	122
Penicillin	a	96	86	94	94	105	83	94
	b	98	98	98	96	110	107	94
Streptomycin	a	98	100	98	98	101	102	100
	b	96	98	96	100	82	98	85
Patulin	a	73	73	92	81	48	63	124
	b	79	86	75	77	64	94	90
Citrinin	a	100	98	100	100	30	96	98
	b	98	98	98	100	25	76	120
Griseofulvin	a	94	98	100	98	18	58	103
	b	87	100	100	100	20	72	104
Griseofulvic acid	a	94	98	100	100	14	71	90
	b	96	100	100	100	14	82	82
Mycophenolic acid	a	92	100	96	100	18	60	96
	b	96	98	100	100	14	60	89
Glutinosin	a	90	98	100	100	15	69	91
	b	96	94	98	96	16	75	89
Gliotoxin	a	83	100	100	100	16	68	101
	b	92	100	100	100	14	73	93
Viridin	a	81	98	100	100	13	69	108
	b	79	100	98	100	15	68	98
Gladiolic acid	a	94	98	100	100	13	84	108
	b	96	100	100	100	13	80	94
Albidin (red pigment)	a	92	98	100	100	11	64	88
	b	94	98	100	100	12	47	89
Alternaric acid	a	90	98	100	98	10	59	95
	b	90	98	100	96	10	55	95

[1] *a* = pH 4·0; *b* = pH 6·0.

TABLE II

Toxicity of Various Antibiotics, 3-Indolylacetic Acid, and Coumarin to White Mustard Seeds

Compound.		% germination				Root growth as % of control		
		25 p.p.m.	5	1	0	25 p.p.m.	5	1
3-Indolylacetic acid	*a*[1]	3	40	68	82	<1	5	10
	b	5	42	78	78	<1	5	16
Coumarin	*a*	80	90	92	92	21	56	81
	b	92	93	90	90	44	95	92
Penicillin	*a*	88	94	90	90	91	110	107
	b	98	83	95	98	103	91	96
Streptomycin	*a*	73	72	78	75	66	80	93
	b	72	75	67	72	93	96	81
Patulin	*a*	82	97	97	92	30	75	115
	b	90	97	97	94	35	71	95
Citrinin	*a*	2	60	78	80	—	33	63
	b	2	68	83	85	—	27	77
Griseofulvin	*a*	38	80	75	80	31	103	92
	b	23	77	82	85	9	78	64
Griseofulvic acid	*a*	7	80	87	90	<1	42	67
	b	5	75	87	93	<1	35	77
Mycophenolic acid	*a*	3	70	85	87	<1	20	57
	b	2	70	82	85	<1	26	47
Glutinosin	*a*	3	52	83	80	<1	10	59
	b	—	25	67	78	—	17	121
Gliotoxin	*a*	—	42	85	93	—	9	51
	b	—	48	82	95	—	25	62
Viridin	*a*	10	60	60	83	2	34	70
	b	7	63	76	85	—	45	62
Gladiolic acid	*a*	7	70	65	85	—	50	88
	b	3	55	62	82	—	52	84
Albidin (red pigment)	*a*	17	85	92	87	—	38	85
	b	12	82	88	92	—	41	74
Alternaric acid	*a*	38	85	87	92	—	10	29
	b	43	80	93	88	—	16	45

[1] *a* = pH 4·0; *b* = pH 6·0.

DISCUSSION

General observations on toxicity

In most cases where root growth was severely inhibited the percentage germination was also reduced. There are a number of exceptions; for instance, gliotoxin reduced root growth of clover to a very low level but percentage germination was scarcely affected. Few compounds reduced the germination of wheat to any marked extent; indolylacetic acid had most effect, but in this case the viability of controls was low, the effect of the inhibitor being consequently enhanced.

None of the antibiotics were as inhibitory as indolylacetic acid, but nearly all were more toxic than coumarin. In general, wheat was less susceptible to the antibiotics than clover or mustard; in this respect the antibiotics resembled

TABLE III

Toxicity of Various Antibiotics, 3-Indolylacetic Acid, and Coumarin to Red Clover Seeds

Compound.		% germination				Root growth as % of control		
		25	5	1	0 p.p.m.	25	5	1 p.p.m.
3-Indolylacetic acid	*a*[1]	1	3	33	70	<1	<1	2
	b	2	15	60	77	<1	<1	8
Coumarin	*a*	100	97	97	98	41	66	94
	b	100	100	98	97	68	82	79
Penicillin	*a*	98	100	98	100	85	102	98
	b	100	98	100	98	109	105	95
Streptomycin	*a*	100	100	98	100	99	95	105
	b	97	95	100	98	104	105	117
Patulin	*a*	97	95	98	97	9	50	85
	b	95	100	97	100	7	50	88
Citrinin	*a*	22	68	73	70	<1	62	73
	b	13	53	72	70	3	39	80
Griseofulvin	*a*	78	95	99	99	23	72	85
	b	83	99	97	97	21	63	87
Griseofulvic acid	*a*	43	75	90	85	13	47	102
	b	53	72	77	90	16	51	93
Mycophenolic acid	*a*	7	35	70	78	1	23	74
	b	12	12	68	78	3	9	72
Glutinosin	*a*	3	55	73	83	—	13	52
	b	5	47	70	85	—	8	35
Gliotoxin	*a*	89	97	100	99	4	25	88
	b	80	99	99	99	3	21	94
Viridin	*a*	85	97	100	97	26	66	94
	b	95	100	97	99	41	88	100
Gladiolic acid	*a*	100	100	100	98	20	58	84
	b	100	100	100	100	34	68	80
Albidin (red pigment)	*a*	87	97	98	100	5	37	87
	b	80	94	97	98	8	61	96
Alternaric acid	*a*	77	98	100	98	17	89	105
	b	67	97	98	100	15	73	86

[1] *a* = pH 4·0; *b* = pH 6·0.

indolylacetic acid but differed from coumarin, which showed little evidence of specificity in these experiments. Audus and Quastel (1947) reported that coumarin was less toxic than indolylacetic acid, but found it to be quite selective in its toxicity. One of the most interesting cases of specificity was that of alternaric acid. This substance, which has been previously shown to be phytotoxic (Brian, Curtis, Hemming, Unwin, and Wright, 1949), though the most toxic of the antibiotics to wheat and mustard, had much less effect than a number of other antibiotics on clover.

Among the more toxic antibiotics were alternaric acid, glutinosin, mycophenolic acid, and gliotoxin. Griseofulvin was only slightly toxic and penicillin and streptomycin had little effect at the concentrations tested. Earlier results obtained with penicillin were conflicting.

Ribeiro (1946) reported inhibition of germination at low concentrations of penicillin. Later it was shown (Smith, 1946; Bustinza and Lopez, 1947; Bein, Signer, and Schopfer, 1947) that this inhibition was due to the presence of impurities of the indolylacetic acid type, commonly found in crude penicillin, and that crystalline penicillin had little effect on germination.

Effect of pH

The pH at which the test was carried out had little effect on the results. In some cases the substances appeared to be more toxic at the lower pH; in the case of gliotoxin and viridin this might well be due to their greater stability at low pH. This general result is very different from that observed when antifungal activity is considered; in that case all the acidic substances are far more active at low pH than at high ones. It has been assumed in such cases that only undissociated molecules penetrate into the fungal cell; this would not appear to be true of seeds.

Inhibition of chlorophyll synthesis by streptomycin

It has already been stated that streptomycin was found to have no significant effect on seeds at the concentrations used. This result was also obtained by Smith (1946) and Ark (1947), but Anderson and Nienow (1947) found that high concentrations were toxic to tomato, radish, and soybean seedlings.

von Euler, Bracco, and Heller (1948) germinated several kinds of seed on filter-paper moistened with concentrated streptomycin solutions and found that chlorophyll synthesis was inhibited, many seedling leaves being completely or partially devoid of chlorophyll. It was thought desirable to repeat these experiments; wheat and radish seeds were germinated in the light on filter-paper moistened with streptomycin solutions at concentrations of 3,000 and 1,500 p.p.m. With wheat there was a marked inhibition of root and shoot growth, especially at the higher concentration, and a greater production of adventitious roots. No seedling was completely white, but the first leaves were green at the tips and white at the bases.

The effect on radish seedlings was more marked. The inhibition of growth was more pronounced and no lateral roots were produced. At 3,000 p.p.m. formation of chlorophyll had been retarded, the cotyledons being completely yellow. At 1,500 p.p.m. the majority of the seedlings were devoid of chlorophyll, but some had patches of green on the cotyledons. A noticeable feature was the abundance of anthocyanin in the cotyledons and hypocotyls of the treated seedlings; in the controls red coloration of the hypocotyls was rare.

An indication of inhibition of chlorophyll synthesis was noticed in mustard seedlings grown on agar containing alternaric acid. When the dishes were left in the light the cotyledons of seedlings grown on agar containing 1 p.p.m. of alternaric acid turned green, but with an outer edge where no chlorophyll was formed.

Formative effects

Indolylacetic acid caused a swelling of the hypocotyl of clover seedlings but no branching of the root-hairs, whereas the former effect was less marked in mustard seedlings but the root-hairs were branched and swollen at the tips. In wheat seedlings the roots were swollen at the tips. Coumarin also caused swelling of the hypocotyl and base of the root of mustard, roots of clover, and coleorhiza of wheat seedlings at the higher concentrations.

Swellings of the coleorhiza was a very general effect on wheat seedlings at the highest concentration of the more toxic antibiotics such as citrinin, albidin, gliotoxin, mycophenolic acid, griseofulvic acid, glutinosin, and alternaric acid. Albidin also caused swelling of the bases of the roots of mustard. The root-tips, tips of the cotyledons, and seed coats of clover became brown where they were in contact with agar containing this antibiotic and at the highest concentration the root-hairs, when present, were very sparse and the cells of the piliferous layer were distorted. A similar effect on clover seedlings in the presence of gladiolic acid was noted. The roots were brown and no root-hairs were formed on the roots which had penetrated the agar containing the antibiotic.

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The Structure and Development of *Himanthalia lorea* (L.) Lyngb.[1]

BY

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With Plate XXV and twelve Figures in the Text

ABSTRACT

The development of *H. lorea* has been followed in detail and comparisons made between comparable stages of development of plants from exposed and from sheltered habitats.

The origin and course of the longitudinal hyphae is traced, and it is found that the distance behind the apex at which they arise varies at different stages of development.

The very pronounced surface growth leads to extreme elongation of the medullary cells which also develop characteristic dilations at the horizontal septa.

In the receptacles the production of the horizontally running hyphae appears to be connected with the formation of central air-spaces. In the older receptacles the cells of these hyphae become extremely swollen and form irregular blocks of pseudo-parenchymatous tissue.

The production of longitudinal hyphae is more marked in plants from more exposed habitats, and the inner cortex has thicker walled cells where the plants have to endure longer periods of intertidal exposure.

HIMANTHALIA LOREA is a widely occurring fucoid of Atlantic shores, with a distribution ranging from the Atlantic coasts of America, Iceland, and the Faroes to the Atlantic coasts of Europe from Norway to Spain (Harvey, 1851, 1871; de Toni, 1895). It occurs on rocky and exposed shores, usually growing near the lower limit of the littoral zone, and often exposed only by the lowest spring tides.

Its striking form has long been recognized, with its juvenile stages resembling a Peziza-cup (Pl. XXV A, 6, 7, 8), later giving rise to the long, dichotomous, strap-like thongs bearing conceptacles along their entire length. These reach a length of between 2 and 10 ft., and some specimens have been reported as reaching a length of 20 ft. (Harvey, 1851).

Rostafinski (1876) described the apical growth of *H. lorea* at various stages of development, but not the anatomical structure. This was investigated in 1910 by Wille, who based his account on a study of tissue differentiation behind the apex of a mature plant, but did not consider developmental stages. Oltmanns (1889) was unsuccessful in his attempts to germinate the oospores

[1] This work formed part of a thesis for the degree of Ph.D. of the University of London.

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of *H. lorea*, but Thuret and Bornet (1878) and Farmer and Williams (1898) both described the early stages of development of the sporelings. Gibb (1937) was able to grow sporelings for a longer period in culture until the plants were 2 mm. high, and was thus able to carry this account a little farther. She also investigated the effect of the position on the shore on the rate of development and on the external form of the thongs. This present investigation consists of a study of the anatomy at various stages of development, and also a consideration of the variation in the anatomical structure in plants from different habitats, neither of which aspects have been considered by the previous workers.

Life-history

Rostafinski (1876) found that the plants he examined required a period of 2 years to reach maturity. Plants developing from eggs fertilized during the winter months had, by the following summer, developed the characteristic button shape, and by the end of the summer were beginning to develop the thongs. These increased in length during the winter and the following year, and by the time that the plants were approximately 2 years old the conceptacles scattered all over the thongs were sexually mature. Gibb (1937) considered the life-history of plants at Port St. Mary in greater detail and found that not all the buttons gave rise to thongs at the end of the first season's growth, but that some of them retained this form for a further year. The number of thongs borne by each button varied from one to four. Gibb (1937) found that they began to develop during the summer, and that during the following autumn and winter they grew in length rather slowly, becoming repeatedly dichotomized. During the following spring they elongated rapidly and the conceptacles became sexually mature during the summer, and after the liberation of the gametes the whole plant usually died. There have, however, been occasional reports of the buttons persisting and giving rise to a second crop of thongs the following season (Turner, 1809; Harvey, 1871). According to Gibb (1937) the production of new receptacles occasionally takes place from the attaching disc. It can thus be seen that the plant requires from 2 to 3 years to complete its life-cycle, the time depending on the age of the button when the receptacles begin to appear.

The peculiar form of the plant has been variously interpreted by earlier workers. There are two main bodies of opinion, the one interpreting the 'button' as the frond and the 'thongs' as the receptacles; and the other considering the 'button' to be a swollen stipe and the 'thongs' to be the fronds bearing the conceptacles scattered all over the surface, as in *Durvillea*. The former view is held by Kützing (1843) and by Harvey (1847, 1871); and the latter by Lyngbye (1819), the founder of the genus, and by Agardh (1848). In 1843 Harvey quotes Hooker as comparing the button of *H. lorea* with the trumpet-like stipe of *Ecklonia buccinalis*; but, as pointed out later by Harvey (1847), whereas in *H. lorea* the thongs and the button develop in succession, in *E. buccinalis* they develop, for a while, at the same time. Wille (1910) and

Gibb (1937) both incline to the view that the plant is sharply divided into vegetative and reproductive regions. Oltmanns (1889), however, does not believe that there is any sharp demarcation between vegetative and fertile regions, but that there is a gradual transition from the one to the other through the basal sterile region of the thong, and compares the condition with that in *Xiphophora chondrophylla*, *Myriodesma*, and particularly with *Hormosira*.

Rostafinski (1876) considered the button-like form of the thallus to be related to the retention of water and the protection of the apex during the ebb. But, as *H. lorea* grows very near low-water level and is therefore exposed for a shorter period than many of the Fucaceae growing at higher levels, there appears to be no need for any special protective measure beyond the mucilage-filled apical groove general in these forms. Oltmanns (1889) believed this form to be merely a special adaptation of the plant to its exposed habitat, enabling it to become firmly established before the development of the elongated thongs. He did not consider that undue weight should be given to the peculiar form of the plant as a feature of systematic importance.

I prefer to consider the button as the vegetative phase of the development of the plant, its form being, as Oltmanns suggested, an adaptation to its exposed habitat. The sterile basal portion of the thong is considered as part of the receptacle, in the same way that the sterile attaching regions of the receptacles of *Ascophyllum*, *Phyllospora*, *Axillaria*, *Scytothalia*, &c., are considered as part of the receptacle. The very long receptacles producing very large numbers of gametes per plant probably compensate for the fact that on account of the exposed habitat only a small proportion of the fertilized eggs succeed in developing, and also for the fact that the plant is only reproductive for a single season before it dies.

Methods

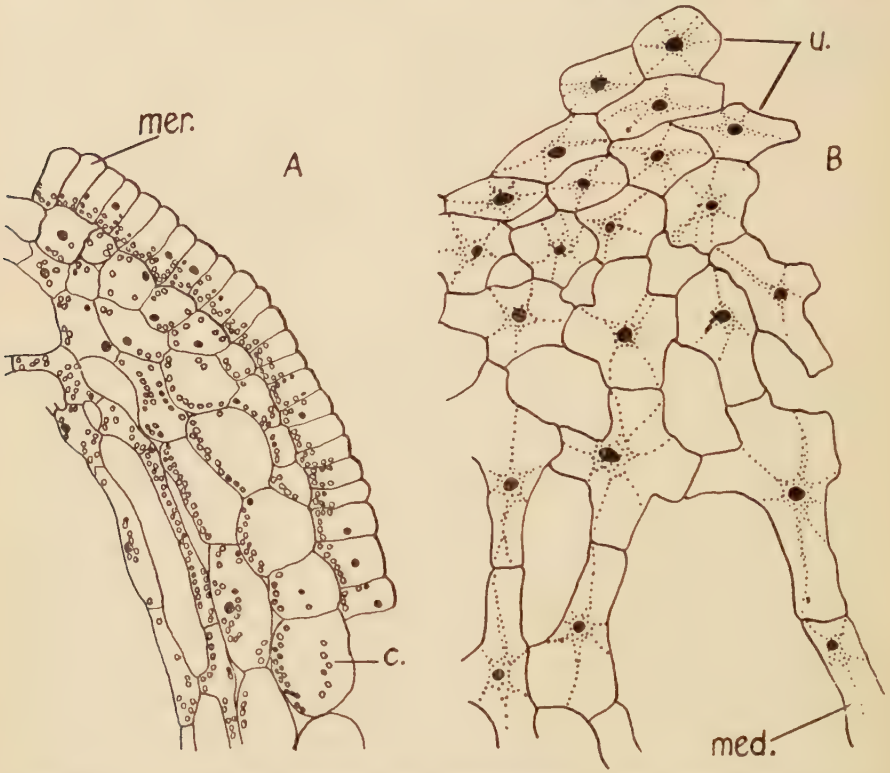
Both fresh and preserved material were examined, by microtome and by hand sections, but principally by the latter. The fixatives used were formalin-acetic-alcohol and Flemming's weaker solution; after fixation the material was stored in 50 per cent. alcohol. Most of the staining was done with Gentian violet, using a 0·05 per cent. solution in 50 per cent. alcohol. The staining was done on a slide using the minimum amount of stain, so that the bulk of it evaporated during the process. First the cytoplasmic contents of the cells stained up blue, and later certain regions of the walls and the mucilage took up a much more reddish tinge. This differential coloration was found to be much more marked if the staining was done slowly in dilute solutions than if more concentrated solutions were used, and appeared to be fairly permanent. Similar results were obtained using aqueous Gentian violet, but the colours were not so clear-cut, possibly due to the better penetration of the alcoholic stain.

The Structure of the Young Sporeling

The youngest plants available—about 2 mm. long—were light in colour, pear-shaped, soft, and filled with a watery fluid. At this stage the tissues are

well developed, and the sporeling resembles very closely a young sporeling of *Fucus*, the only important difference being that in this case the sporeling is very much distended and filled with fluid.

A dot at the distal end marks the position of the apical pit, which is filled with mucilage and lined with a meristoderm of elongated cells. At the base



TEXT-FIG. 1. L.S. young sporeling 2 mm. long showing (A) the meristoderm (*mer*) and the cortex (*c*), and (B) the separation of the medullary cells behind the apex to form longitudinally running filaments (*med*). *u* = undifferentiated cells behind the apex.

of the depression is a single, clearly marked apical cell, which, as was earlier described by Rostafinski (1876), is biconvex in longitudinal section and triangular in transverse section.

The sporeling is bounded by a single layer of cells, slightly elongated in the direction perpendicular to the surface. These cells contain a single nucleus and a number of discoid plastids which are aggregated towards the inner margins of the cells (Text-fig. 1 A, *mer*). Inside this meristoderm is a cortex about three cells in depth, the size of the cells increasing towards the interior. The cells become slightly elongated in the direction of the longitudinal axis towards the interior, and also become somewhat separated from each other (Text-fig. 1 A, *c*). These cells also contain fairly numerous plastids. The fluid occupying the central region supports a few widely separated filaments

of medullary cells. In the centre the cells are considerably elongated in the longitudinal direction, and are arranged in regular, uniseriate filaments with occasional horizontal pit-connexions between the cells. Towards the cortex the cells are less elongated with more horizontal pit-connexions, and grade imperceptibly into the cortex. The cells of the medulla are uninucleate and contain few plastids. Immediately behind the apical cell is a region of small, regular cells, with dense, granular, and deeply staining contents (Text-fig. 1 B, *u*), and as the distance from the apex increases, stages in the elongation of these cells to form the medullary filaments can be clearly seen (Text-fig. 1 B, *med*).

Even in these very young plants a number of hyphae are present. In many cases these arise from the plate of cells immediately behind the apex (Text-fig. 2 A) as small outgrowths, later being cut off from the parent cell by a transverse wall. The hyphae have thin, refractive walls, and towards their tips have a small diameter and densely staining contents (Text-fig. 2 A, *d*; Pl. XXV B, *hy*). As the hyphae increase in length they become septate and in the older parts increase in diameter and their contents become less dense, so that the older regions of the hyphae are scarcely distinguishable from the medullary cells. Farther away from the apex the hyphae also arise as lateral outgrowths from the medullary cells, by a process resembling evection. At this stage all the hyphae run longitudinally.

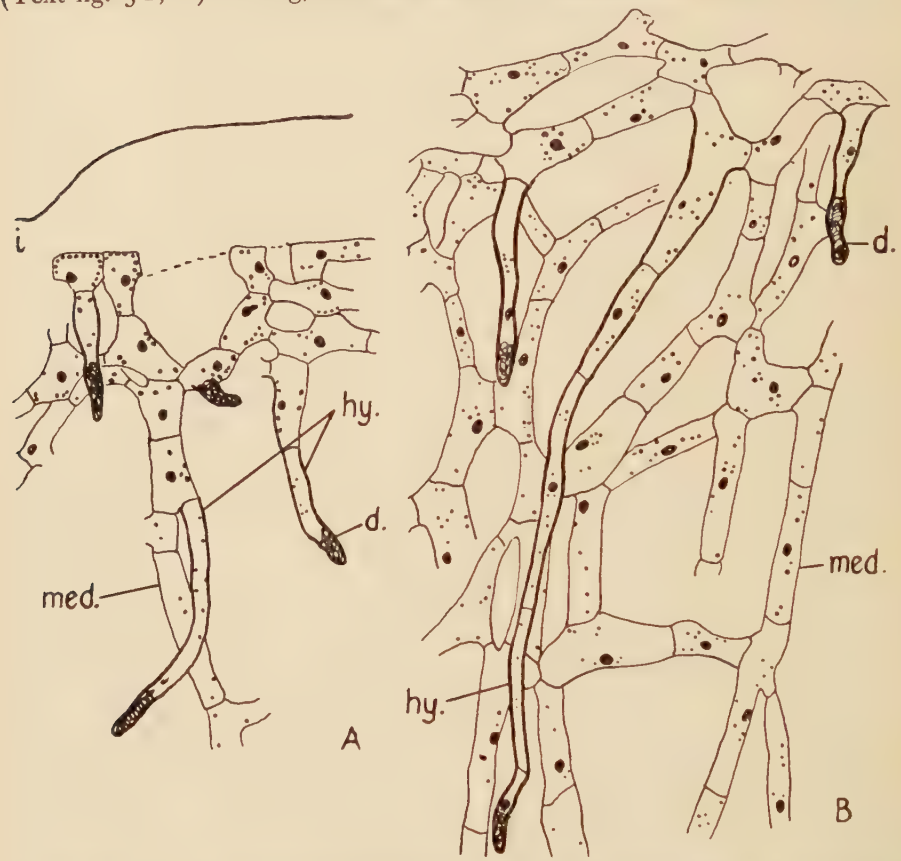
The development of the 'button'

At an early stage the sporeling begins to flatten out and form the characteristic 'button'. This involves a change from a soft, pear-shaped and fluid-filled sporeling (Pl. XXV A, 1–4) to a tough and leathery button, solid throughout.

The early stages of this process are easily recognized as they consist of the formation of a firm and flat top to the still conical and fluid-filled sporeling (Pl. XXV A, 5). The tougher nature of this top can be seen on drying, when the top remains flat whilst the sides collapse and cave in. At this stage there is a considerable increase in the thickness of the cortical region as the result of tangential divisions of the meristoderm. At the same time there is an increase in the thickness of the cell walls in the inner cortex, this thickness increasing in degree and extent with the age of the plant.

During the early stages of flattening the meristoderm cells of the upper surface become rather long and narrow, indicating that they are dividing actively by anticlinal walls. These divisions are taking place in both the radial and tangential directions, and are responsible for the initiation of the flattening. As the flattening proceeds and the button form becomes established, the meristoderm cells of the upper surface become extremely long and narrow (Text-fig. 3 A, *m.u.*), whilst those of the lower surface remain comparatively short and broad (Text-fig. 3 A, *m.l.*), their breadth increasing as the flattened portion narrows into the stipe. This shows that active divisions causing the flattening are restricted to the upper surface.

As button formation continues, the increase in depth of the cortical region is carried still farther, and when the button is fully developed, four regions can be distinguished in this outer region. On the outside is the meristoderm (Text-fig. 3 B, *m*) of long, narrow cells, and beneath this are two or three



TEXT-FIG. 2. L.S. young sporeling showing origin and structure of the longitudinally running hyphae.

(A) L.S. 2 mm. long sporeling with hyphae originating from cortical cells close to the apex, and from the medullary cells.

(B) Apical region of slightly older sporeling. In both the hyphae are outlined with a slightly firmer line than the primary cells.

d = dense contents at tip of hypha; *hy* = hyphae; *i* = position of apical cell; other letters as in Text-fig. 1.

layers of small, thin-walled cells containing numerous plastids (Text-fig. 3 B, *s.c.*), and which have recently been derived from the meristoderm, as can be seen from their arrangement. Internally to this is a region about three cells deep, composed of large cells whose walls are already beginning to show signs of thickening (Text-fig. 3 B, *o.c.*). Finally there is a region, four or five cells in depth, of large cells elongated in the radial direction, with thick walls and sparse contents, the thickness of the walls increasing towards the centre

(Text-fig. 3 B, *i.c.*). There are frequent thin-walled pit-connexions between the cells, and occasional thin secondary walls.

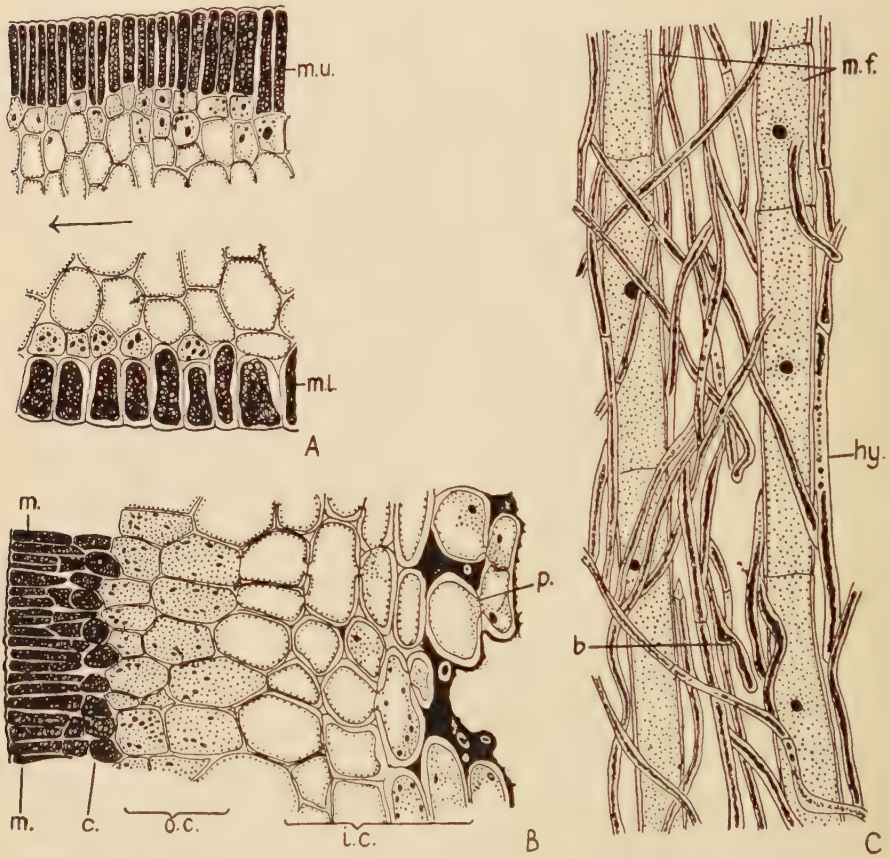
Wille (1910) only describes two zones of cells in this region, an outer zone of assimilatory cells and an inner one of mechanical cells. The assimilatory zone no doubt corresponds with the meristoderm and the few cells beneath it, and the mechanical cells with the inner thick-walled cortical cells, which are also described by Wille as being divided by secondary walls. Wille describes the thick-walled cells as containing a great deal of fucosan, and ascribes to them a storage as well as mechanical function.

The edge of the button and the lower surface show a very similar development to the upper surface, but the amount of activity of the meristoderm seems to diminish towards the lower surface. Here, and in the stipe, the meristoderm cells are much broader and less elongated than on the upper surface, and possess very thick, brown outer walls showing a lamellated structure, as described by Wille (1910). In this region the cortex is only two or three cells in depth—i.e. no more extensive than in the young sporeling —and there is no appreciable increase in the size of the individual cells, and their walls are scarcely thickened.

At first the thick-walled cortical cells may form a tough outer region enclosing a medulla which contains relatively few cells supported in a watery fluid. But, as development proceeds, very large numbers of hyphae are produced, both from the medullary and from the inner cortical cells. At their tips these hyphae resemble those of the young sporeling, but develop slightly thicker, highly refractive walls. They grow downwards towards the base of the stipe, becoming septate as they increase in length: and in many cases the contents appear to consist of deeply staining droplets (Text-fig. 3 C). At intervals there are bulbous swellings in the walls of the hyphae, similar to those described by Wille (1910) in the 'primäre Leitungszellen', and at intervals they are seen to branch (Text-fig. 3 C, *b*; Pl. XXV C) by a process resembling evection. The narrow, deeply staining cells of the hyphae can always be readily distinguished from the much larger and less deeply staining filaments of the primary medullary cells (Text-fig. 3 C; Pl. XXV C). Wille (1910) believes that the hyphae may perform a storage function and describes them as establishing connexions with the medullary cells, but I have not seen this. Very soon these hyphae greatly outnumber the primary medullary cells, and, interweaving with each other and with the medullary cells, form a firm tissue occupying the central regions of the button, which thus becomes solid throughout. When this condition has been produced, longitudinal sections of the stipe show the filaments of large medullary cells widely separated by the closely interweaving, small-celled hyphae.

Meanwhile the outer walls of the meristoderm cells of the stipe have become extremely thickened on all their walls, but particularly on their outer walls, and lamellations similar to those described by Wille (1910) are clearly visible on the walls (Text-fig. 4 A). In places on the inner tangential walls there remain thin-walled pits between the meristoderm and the outermost

cortical cells. The walls of the meristoderm increase in thickness, and the thickening also extends to the walls of the outer cortical cells. The contents of the meristoderm cells eventually die, becoming a dark brown in the process, and the cells rub off (Text-fig. 4 B). The thick-walled cortical cells are



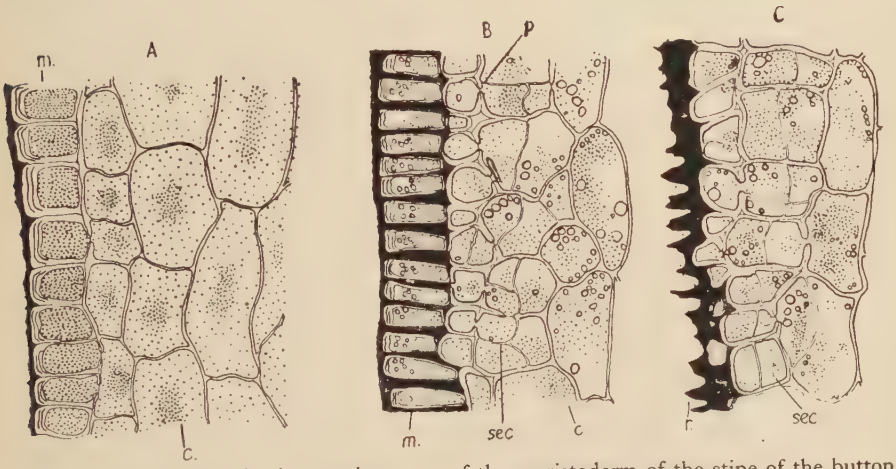
TEXT-FIG. 3. (A) V.S. young plant in which button formation is fairly well established, showing the meristoderm of the lower surface (*m.l.*) in which few, if any, divisions have taken place; and the meristoderm of the upper surface (*m.u.*) where frequent anticlinal divisions have taken place. The arrow points to the centre of the button.

(B) V.S. well-developed button, showing the meristoderm (*m*) which is dividing both anticlinally and periclinally; the outer cortex of large, thin-walled cells (*o.c.*); and the inner cortex of thick-walled cells with thin-walled pits (*p*) in their tangential walls.

(C) R.L.S. of the stipe of the button, showing the filaments of large medullary cells (*m.f.*) and the interweaving hyphae (*hy*); *b* = point of branching of a hypha.

thus brought into an external position (Text-fig. 4 C). Meanwhile the thickening of the walls of the cortical region has extended farther inwards, and the cells have begun to divide by thin walls, mostly in the periclinal direction (Text-fig. 4 B and C, *sec*). Usually only two or three divisions take place in each cell, so that each primary cell is replaced by a short radial row of cells.

Occasionally a division may occur in the anticlinal direction. During the process a change takes place in the nature of the contents of the cortical cells, which come to contain a large number of highly refractive droplets. Wille describes the degeneration of the outer layer and the development of thick walls in the second layer, but does not mention any segmentation in the cortex.



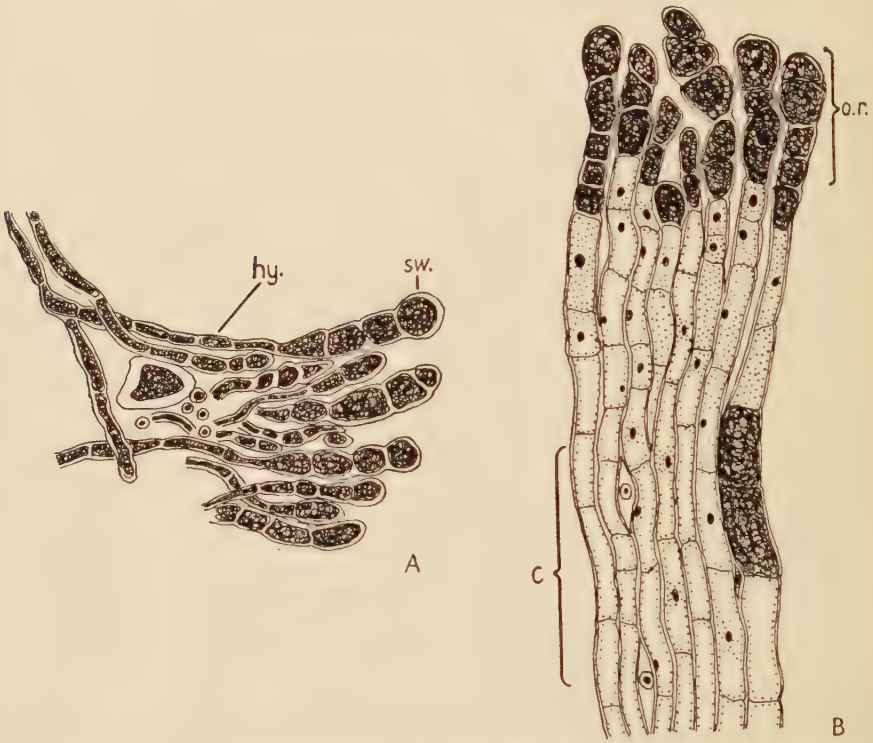
TEXT-FIG. 4. Stages in the wearing away of the meristoderm of the stipe of the button, and its replacement by the outer layers of the cortex. (A) L.S. through the stipe before the process begins, showing the very thick walls of the meristoderm with lamellate structure, and with thin-walled pits between them and the outer cortical cells. (B) The beginning of the degeneration of the meristoderm. A few thin walls—mainly in the tangential direction—have been formed in the outer cortex. The walls of the cortical cells begin to show thickening. (C) The final stage. The meristoderm is now completely worn away exposing the outermost cortical cells which are now thick walled and protective. There are now rather more secondary divisions visible in the rest of the cortex.

p = pit; *r* = remains of meristoderm cells; *sec* = secondary walls.

Thus, in the old stipes, the protective function of the meristoderm is taken over by the cortex. The secondary divisions of the cortex are not here sufficient to give rise to any appreciable increase in the diameter of the stipe. Similar, but usually more pronounced, secondary activity of the cortex has long been recognized in other members of the Fucales. In *Fucus*, for example, it was first seen by Reinke (1876), who described the outermost layer as losing its meristematic activity and being sloughed off like the epidermis of some dicotyledons, the inner cortical cells then dividing tangentially into radial rows of cells forming a secondary cortex, in contrast with the primary cortex developed from the 'epidermis' (Reinke, p. 334). This secondary cortex comprises the greater part of the stipe of old plants, and may be as many as twenty cells in depth, the depth diminishing towards the base.

Oltmanns (1889) also describes this secondary division in *F. vesiculosus*, and Pennington (1937) in *F. serratus*, *F. vesiculosus*, and *F. spiralis*. Pennington found that, whereas in *F. serratus* and *F. vesiculosus* the divisions occurred simultaneously in several layers of cells towards the outer boundary of the

cortex, in *F. spiralis* they were restricted to a single layer towards the inner margin of the cortex. Moss (1950) describes this process in *F. vesiculosus* in greater detail than the previous writers. Hansteen (1892) describes the process in *Pelvetia*, and I have seen it occurring throughout the cortex of the old regions of the axis of *Halidrys siliquosa*.



TEXT-FIG. 5. (A) R.L.S. stipe at the region of transition to disc structure, showing the down-growing hyphae (*hy*) turning outwards and forming swollen terminal cells (*sw*) with dense contents.

(B) V.S. outer region of the disc (upper surface) showing the outer region of deeply staining cells (*o.r.*) and the inner, regular, pseudo-parenchymatous tissue (*c*) formed from the closely packed hyphae.

Thus this secondary meristematic activity of the cortex of the stipe is not unusual amongst the Fucales, but in the stipe of *H. lorea* it does not occur to anything like the extent it does in the other longer-lived fucoids where it has been studied.

Towards the base of the stipe many of the longitudinally running hyphae turn outwards to the periphery and frequently become much branched. These outgrowing hyphae are characterized by their deeply staining contents. When they reach the exterior, a very swollen terminal cell is produced (Text-fig. 5 A) and the hyphae become segmented from the apex backwards. This segmentation is accompanied by an increase in diameter of the individual cells, and so, from the numerous, closely packed hyphae a compact, pseudo-

parenchymatous cortex is produced (Text-fig. 5 B; Pl. XXV D). In this manner a roughly circular disc is produced which increases in size by the apical growth of the hyphae. Wille (1910) described this manner of disc formation in *H. lorea*, and similar disc formation has been described in other members of the Fucales (Oltmanns, 1889; Rees, 1932; Moss, 1950). At its lower edge, where it comes into contact with the substrate, the cells of the disc segment and form a very regularly arranged tissue. Uneven growth rates at the lower margin cause the disc to fit closely into any irregularities of the substrate, giving a firm grip. The lowermost cells in contact with the substrate usually have rather dense contents.

The central region of the disc is occupied by longitudinally running hyphae continuous with those of the stipe. These hyphae are closely interwoven and are often very swollen, and recall the central swollen hyphae of the stipe and disc of *Durvillea antarctica*. Wille's statement that the lower ends of the medullary filaments grow out into hyphae which penetrate down into the disc is very difficult to verify.

Some of these developments in the button take place before, and some after, the production of the receptacles. The receptacles do not normally develop until the flattened form of the button is well established (cf. Gibb, 1937), but the formation of the solid core may not be completed until the receptacles are well developed. The wearing away of the meristoderm also may not occur until a late stage of development.

Very few further changes take place in the structure of the buttons. In the older buttons the meristoderm of the upper surface divides repeatedly by both anticlinal and periclinal walls giving rise to a small celled tissue, about five cells deep, composed of regular rows of cells arranged perpendicular to the upper surface. Wille (1910) describes how the outer cells of the upper surface become extremely elongated to a length of about twenty times their depth before dividing into a series of five or six cells, but I have not seen this condition.

The Receptacles

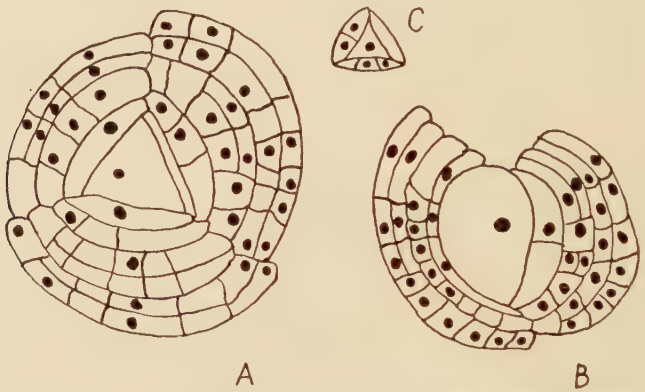
The appearance of the receptacles is commonly preceded by the dichotomy of the apical cell, the two resulting apical grooves becoming slightly elevated by the very active divisions of the intervening meristoderm cells (Rostafinski, 1876). At the same time the depth of the cortex of thick-walled cells becomes greater in the region of the apical grooves, and the medullary cells do not become separated by the gelatinization of their walls, but form a thick-walled tissue with many horizontal pit-connexions between the cells. Abundant hyphae running down into the stipe arise from the cells of this region. Thus there is formed a central, firm tissue above which the receptacles arise as the result of the increased activity of the apical cell.

(*a*) *The very young receptacles*

During the very early stages of its development—i.e. up to about 2 mm. in length—the receptacle is rounded in transverse section, and has a slightly

elongated apical groove at the base of which is a single, large, three-sided apical cell (Text-fig. 6 A and B).

When it first becomes visible the receptacle is bounded externally by a meristoderm of considerably elongated cells, similar to the elongated meristoderm cells seen by Fritsch (1945) in the apical groove of *Halidrys siliquosa*. This form is indicative of active anticlinal divisions assisting in the elongation of the receptacle. Beneath the meristoderm are two or three layers of small, thin-walled cortical cells derived from the meristoderm, and inside these a broader zone about nine or ten cells deep, of thicker walled cortical cells,



TEXT-FIG. 6. (A and B). T.S. and L.S. of apical cells of buttons just beginning to give rise to receptacles.
(C) T.S. apical cell of a receptacle 8 in. long. All to the same scale.

rather irregularly arranged and derived from the segmentation of the apical cell. In the centre is the medulla composed of cells with slightly thinner walls than those of the cortex and slightly elongated in the direction of the longitudinal axis. These cells are continuous with the medullary cells of the button, and form a firm central core to the base of the young receptacle.

The whole apex resembles that of *H. siliquosa* (Fritsch, 1945), and the segmentation of the apical cell and the origin of the primary tissues follows the same course. As in *H. siliquosa*, a striking feature is the rapidity with which the cortical cells become thick-walled, even right up to the apical groove and in the lobes around it. As in *H. siliquosa*, thinner septa indicate that these cells are still dividing actively. A characteristic of these thick-walled cells is their staining reaction with Gentian violet. Whereas the walls of the outer and thinner walled cells do not readily take up the Gentian violet, the central portion of the walls of these cells rapidly stains an intense reddish-purple, thus affording a very clear and easy means of recognition in both transverse and longitudinal section. This staining characteristic is seen in the inner cortical cells throughout the whole life-history of the receptacle.

Characteristic of the very young receptacle are the hyphae produced from the inner cortical and the medullary cells. They are produced right up to the

apex, arising even from the plate of undifferentiated cells immediately behind the apex, and from the cortical cells occupying the lobes surrounding the apical groove. These hyphae have a small diameter, with deeply staining contents and a nucleus at their tips, and have highly refractive walls. They run downwards between the primary cells into the button.

As the receptacle increases in length, there are various changes in its organization. The most evident of these is the change from radial to bilateral symmetry which accompanies a change in form of the apical groove, which becomes elongated in the direction *perpendicular to the plane of flattening* of the receptacle. In those intertidal Fucaceae which have been investigated the groove is usually parallel with the plane of flattening, but in *Halidrys* it is also perpendicular to the plane of flattening.

At an early stage in receptacle formation some of the meristoderm cells lining the apical groove grow out into short, mucilage-producing hairs with slightly swollen terminal cells. Similar hairs are only described in the apical grooves of the mature plants of two other species, in *Bifurcaria laevigata* (Laing, 1941) and in *Xiphophora chondrophylla* (Mitchell, 1941). In both *H. lorea* (Oltmanns, 1889; Nienburg, 1913) and in *B. laevigata* (Laing, 1941) these hairs have been shown to be closely connected with the initiation of the conceptacle. This may explain why in *H. lorea* they are not present before the receptacles develop. There is no information available as to whether they occur in the young fronds of *B. laevigata* before receptacle production begins. They are not figured in *X. chondrophylla* by Heine (1932).

These lining hairs differ from the apical tufts of hairs reported in the young sporelings of some members of the Fucaceae; in *Fucus* (Oltmanns, 1889; Nienburg, 1929, 1931); in *Pelvetia* (Oltmanns, 1889); in *Xiphophora* (Heine, 1932), and in *Hormosira* (Osborn, 1948). In the young sporeling the apical hairs, which are usually shed at an early stage, are long, basally growing, ectocarpoid hairs occupying only a small area in the region of the apical cell, and there is no evidence that they produce mucilage, as do the lining hairs of the apical groove of the receptacle of *H. lorea*. There is no record of any such hairs in the apical groove of the young sporeling of *H. lorea*. Thuret and Bornet (1878) and Oltmanns (1889) were not very successful in their attempts to grow the sporelings in culture and give no detailed account of the early stages of development. Gibb (1937) was more successful and grew sporelings up to a length of 2 mm., but did not describe any apical hairs. I was not successful in my attempts to grow the sporelings, and the youngest plants I examined—about 2 mm. in length—possessed no apical hairs, nor were any present at any stage of the development of the button. In *H. lorea* the appearance of the short hairs lining the groove coincides with the beginning of receptacle development, and they appear to be in no way connected with its growth in length. *X. chondrophylla* possesses both the long basally growing hairs in the sporeling stage (Heine, 1932) and the shorter type of hair in the adult plant (Mitchell, 1941).

By the time that the receptacle is 2 or 3 mm. in length, slightly more

internal differentiation can be seen than at its first appearance. In the extreme basal region the medullary cells remain thick-walled and compact, but immediately behind the apex the cells are thin-walled, roughly cubical in shape with prominent nuclei, and are separated by mucilage into longitudinally running, uniseriate filaments of cells, connected by numerous horizontal pit-connexions. This amount of differentiation is visible before there is any sign of conceptacle development.

(*b*) *The rapidly elongating receptacle*

The receptacles soon become repeatedly branched and pass through a phase of active elongation (cf. p. 502). The conceptacles begin to develop, but not beyond the stage of the differentiation of the basally growing hairs which project through the ostioles. The oogonia and antheridia do not develop whilst this most active elongation is taking place. During this stage considerable internal differentiation takes place very rapidly, often being complete in receptacles only a few centimetres long, and very close behind the apex.

The cells of the meristoderm are elongated in the direction perpendicular to the surface and contain numerous plastids. They are obviously dividing actively by anticlinal walls, but not so actively by periclinal walls.

Beneath the meristoderm there are usually two or three layers of small cells containing numerous plastids and which constitute the secondary cortex (Text-fig. 11 A, *s.c.*). Internally to this lies the rest of the cortex, which is composed of larger cells with fewer plastids, and which can be divided into an inner and an outer cortex. The outer cells are almost isodiametric and thin-walled, whilst towards the interior, as a result of surface divisions, the cells become progressively more elongated in the direction of the longitudinal axis. As the cells become more elongated they often become divided by thin, horizontal septa into short, vertical series of cells (Text-fig. 11 A, *s*). The walls of these inner cells are usually thickened, the thickening being laid down in concentric lamellae.

At the innermost limits of this inner cortex there are a few layers of cells of slightly different appearance. Instead of having tapering end walls like the neighbouring cells, they have flattened end walls and are arranged in regular longitudinal filaments (Text-fig. 11 A, *f*) like the cells of the medulla. Their vertical walls are thickened, but the horizontal walls usually remain thin. There are frequent thin-walled pits in the vertical walls, as there are also in the vertical walls of the tapering cells. These cells also become divided by thin horizontal septa. Wille (1910) describes the thin regions as being perforated by very fine pores, and believes that as well as serving a mechanical function these cells are also used for conduction in the horizontal direction. I have not been able to detect actual perforations, not even after swelling such as applied by Hick (1885) to demonstrate the perforations in *Ascophyllum nodosum*, but where the treatment causes the protoplast to retract, it usually remains in close contact with the horizontal walls and the pits, indicating that there may be perforations in these regions.

As the plant increases in age, the periclinal divisions of the meristoderm add further cells to the outside of the cortex. With increasing age the thickness of the walls of the inner cortical cells increases (cf. Text-fig. 11 A and B), and the thickening also extends farther out into the cortex so that a wider zone of thick-walled cells is produced. As the thickening increases, the successive lamellae can be very clearly seen.

In the medullary region immediately behind the apical cell there is a region of undifferentiated cells with dense granular contents. These separate out into the longitudinally running filaments of medullary cells connected at intervals by thin-walled pits. These pits become considerably stretched as the distance from the apex increase, owing to the abundant production of intercellular mucilage. These medullary cells have thin walls and a conspicuous central nucleus.

Wille (1910) describes the inner products of the segmentation of the apical cell as dividing repeatedly to form short, longitudinal series of cells. These series of cells are described as separated and irregularly arranged, but remaining in contact through horizontal pit-connexions, thus forming a complicated network. Indications of such divisions are frequently seen in the immediate neighbourhood of the apex (Text-fig. 7 A), but there is very regular formation of longitudinal series of cells—similar to that seen in *Fucus* and *Ascophyllum* —which was not seen by Wille.

Frequently, however, the medulla appears to consist of a network of cells, apparently resulting from the branching and anastomosis of the medullary cells. In the neighbourhood of the apex branches can be seen arising as outgrowths from the medullary cells. These branches grow both towards the base of the receptacle (Text-fig. 7 C) and towards the apex (Text-fig. 7 D and E). These outgrowths are of the same diameter as the medullary cells from which they arise, and resemble them in every respect. They grow apically and become divided by transverse walls into short series of cells. In places they appear to form anastomoses with other medullary filaments (Text-fig. 7 F). Moss (1950) describes similar branching of the medullary cells in *Fucus vesiculosus*, but only in the very swollen receptacles, and Osborn (1948) also describes it in *Hormosira Banksii*.

Although there is much 'formation of cross connections by the extension of the pits left in these (i.e. gelatinized) longitudinal walls' (Fritsch, 1945*a*, p. 358), and these connexions are later pulled out of their horizontal course by unequal rates of growth in adjacent filaments, thus giving an appearance of reticulation, this alone is insufficient to explain all the features of reticulation in the medulla of *H. lorea*. Appearances such as those seen in Text-figs. 7 F and G may be in part due to the breakdown of the process of the regular formation of longitudinal series of cells at the apex, a single series being replaced by two filaments, as at the point *P* in Text-fig. 7 F, or the reverse process may explain the appearance of the central filament in Text-fig. 7 G. This latter appearance may equally well have been arrived at, however, by the septation of a primary pit-connexion, or by anastomosis, as in F. In a few

TEXT-FIG. 7. Cells of the medulla from immediately behind the apex of an actively elongating receptacle 5 cm. long. In all cases the cells are arranged in longitudinally running filaments and the outermost layer of the cell wall appears very deeply staining—indicated by a heavy line.

(A) Cells very close to the apex, showing a primary pit between cells of adjacent filaments. The presence of two nuclei in some of the cells, and the outlines of the walls, indicate that cell divisions are still occurring in these cells.

(B) Small lateral protuberances have been produced by adjacent cells and appear to be establishing a secondary pit.

(C) Branching of a medullary filament, the branch growing away from the apex.

(D and E) The branches growing towards the apex.

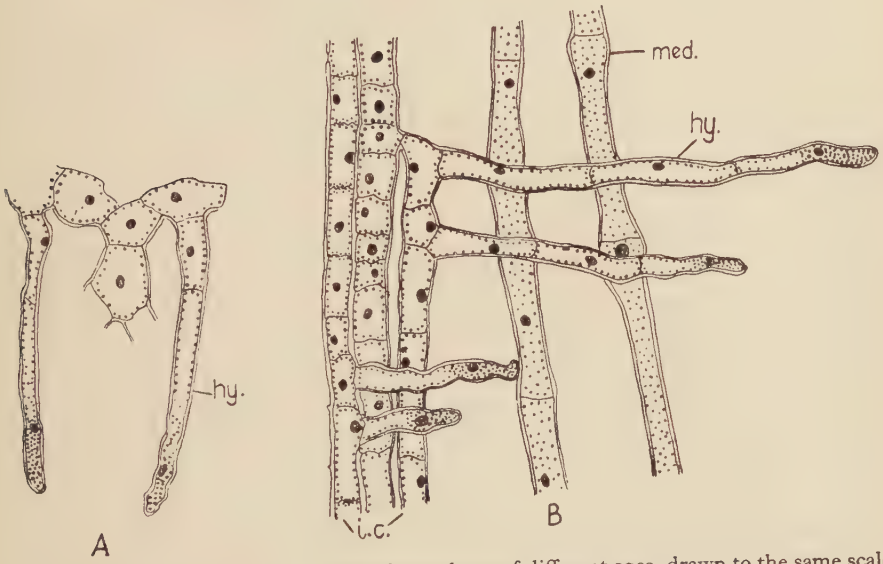
(F) At *P* the left-hand filament is replaced by two filaments, possibly as the result of anticlinical divisions at the apex. At the point *Q*, the central filament seems to have branched as in (C), and at the point *R* this branch is fusing with the right-hand side filament.

(G) The arrangement of the filaments may have been arrived at by a process similar to that seen in (F), or possibly by the septation of a pit connexion at the point *T*.

places there are also indications of the formation of secondary pits (Text-fig. 7 B), in a manner similar to that seen in the Laminariales (Killian, 1911). As the distance from the apex increases, the medullary cells become elongated in the direction of the longitudinal axis until their length is many times their breadth.

A striking difference between the structure of the apex of the actively elongating receptacle and that at its first appearance is that now no longitudin-

ally running hyphae are produced in the apical region. The only longitudinally running hyphae present are those in the extreme basal region which were produced as soon as that part of the receptacle was formed. This shows that the distance behind the apex at which hyphae are present in older receptacles is not necessarily an indication of their developmental sequence, since the activity of the apex may vary at different stages of development. As the age of the receptacle increases, many more longitudinally running



TEXT-FIG. 8. Comparison of hyphae from plants of different ages, drawn to the same scale.
(A) Hyphae from immediately behind the apex of a sporeling 2 mm. long.
(B) Horizontal hyphae from immediately behind the apex of a receptacle 12 cm. long.

hyphae are produced, and in some cases they may eventually be produced within a few millimetres of the apex. There is, however, no question of these first-formed hyphae being produced *in response* to strains in the basal region of the receptacle, since they were present *before* any such strains could occur. This point is of interest in comparison with *Fucus vesiculosus* where Moss (1950) finds that hyphae are found closer to the apex in mature plants from the more exposed habitats. It would be of interest to compare the levels at which the hyphae occur in the sporelings. Pennington (1937) states that in sporelings 'the first descending hyphae are formed much nearer the apex than they arise in the mature plant'.

Very close to the apex of these young receptacles of *H. lorea*—usually within 1 mm.—*horizontally running hyphae* arise as outgrowths from the inner walls of the cortical cells (Text-fig. 8 B). These grow principally, but not exclusively, in the plane perpendicular to the plane of flattening. These hyphae are thin-walled and at their tips possess deeply staining contents and a nucleus. They grow apically and soon become divided by transverse septa

into a number of cells, but they never become very long. In their early stages of development they closely resemble the longitudinal hyphae of the young sporelings (Text-fig. 8 A). As they become older their diameter increases and they soon become almost indistinguishable from the cortical cells from which they arise.

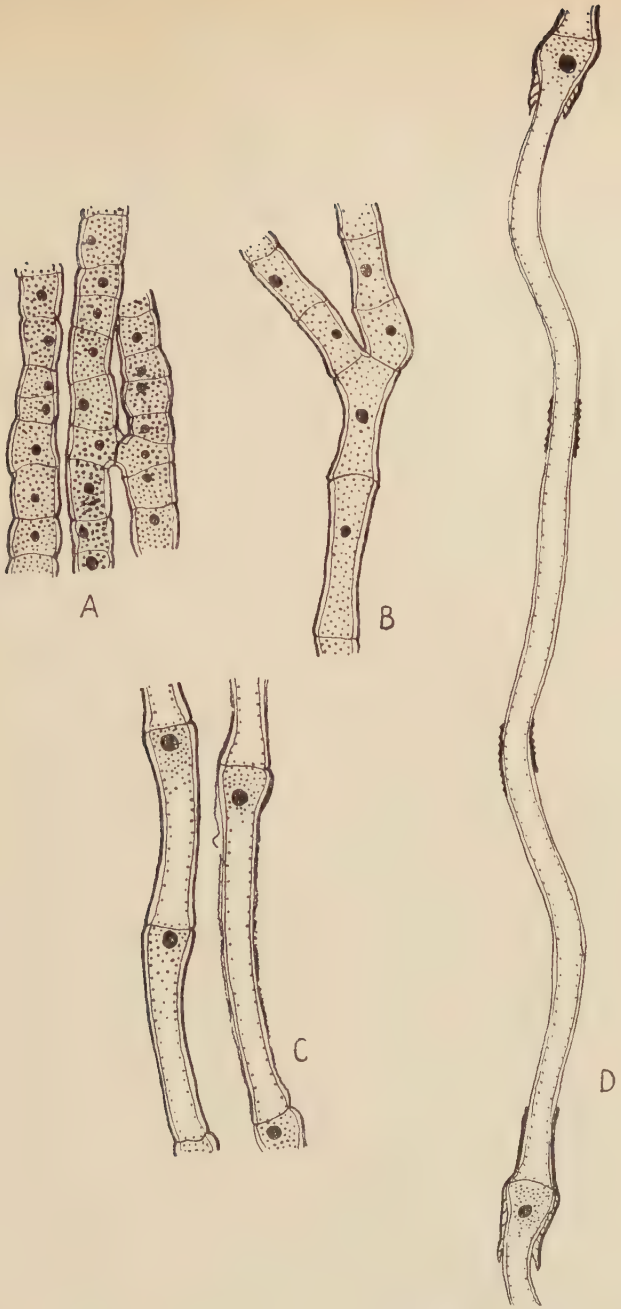
Wille (1910) describes these horizontal hyphae as having perforated transverse septa and forming anastomoses with the medullary cells, but I have not seen either of these features. He believed that they served for conduction in the horizontal direction from the 'storage cells' to the 'conducting cells'. Horizontally running hyphae are not recorded elsewhere in the Fucales, except in *Durvillea antarctica* (Naylor, 1949), where they are concerned with the formation of the diaphragms separating the central air chambers. Similar short, horizontally running hyphae are commonly produced in the Laminariales.

In the basal region of the receptacle the medullary cells are shorter and thicker walled. There is little mucilage in this region, and the presence of numerous horizontal pits gives an irregular appearance to the cells.

As the rapid elongation continues, it does not appear to be accompanied by much further differentiation of new tissues, although there may be some addition to the outer cortex as the result of periclinal divisions of the meristoderm. The most important changes are the increased stretching of the inner tissues of the cortex and of the medulla due to the surface growth.

In the case of the medulla this elongation is carried to extreme lengths, the cells often becoming stretched until their length is about 30 times their diameter. Immediately behind the apex the cells appear square in longitudinal section, with dense, granular contents and a central nucleus (Text-fig. 9 A). When the elongation first begins the cell contents remain evenly distributed, but very soon the nucleus can be seen to be nearer the upper end of the cell than the lower (Text-fig. 9 B). When the length of the cell reaches about four times its diameter, the cytoplasm is no longer evenly distributed throughout the cell, but is mainly restricted to the upper end of the cell, and the nucleus is immediately beneath the upper transverse wall (Text-fig. 9 C, left-hand filament). Up to this stage of development the cell retains its original diameter throughout its length, but now, as the elongation increases, the diameter of the cell decreases in its middle region. Thus the region immediately below the transverse wall where the bulk of the protoplast is accommodated appears as a slight dilation (Text-fig. 9 C, right-hand filament). In this way an appearance is obtained resembling the trumpet hyphae of the Laminariales, this appearance becoming more marked as the elongation continues (Text-fig. 9 D). These very much elongated cells resemble very closely the sieve tubes of *Laminaria Cloustoni* figured by Wille (1910).

The early stages of the elongation of the medullary cells (Text-fig. 9 B) are probably due to turgor, and are accompanied by the deposition of some new material in the walls. At a later stage (Text-fig. 9 C), rapid surface growth causes the passive stretching of these cells, resulting in the characteristic



TEXT-FIG. 9. Stages in the elongation of the medullary cell. A, B, and C drawn from the same apex as previous figure; D from the apex of a 16 cm. long receptacle.

(A) Cells immediately behind the apex.

(B) Between 1 and 2 mm. behind the apex, already showing considerable elongation.

(C) Between 2 and 3 mm. behind the apex. In the left-hand filament the cytoplasm and nuclei are at the upper end of the cell. In the right-hand filament, which is nearer the centre of the receptacle, the elongation is still greater, and the rupture of the outermost layer of the cell wall has begun, and the slight dilation of the upper end of the cell can be seen. In (D) the elongation has been carried to its full extent. The remains of the ruptured outer layer of the cell wall can be seen in the region of the transverse wall, and at intervals along the length of the cell.

Scale = ×250.

attenuation of the middle regions of the cells (cf. Küster, 1889) where there are no supporting transverse walls, a feature which becomes the more marked as the elongation continues. Despite this extreme elongation, the walls do not become markedly thinner, so possibly new material is being formed throughout the whole process.

It is of interest that the outermost layer of the wall does not extend to the full length of the greatly elongated cells. In the early stages of development an outer layer of the wall, deeply staining with Gentian violet, can be seen around the cell. This is indicated in Text-fig. 9 by the firm outer line. As the elongation proceeds, this outer layer becomes ruptured, usually at the stage when the protoplast is becoming restricted to the upper end of the cell, and very often just below the protoplast. This outer layer usually remains intact for a short distance on either side of the transverse wall, and in the extremely elongated cells can often be seen surrounding the dilation containing the protoplast, and as fragments along the length of the cell (Text-fig. 9 D). This rupture possibly marks the beginning of the active surface growth, and indicates that either the outer layer of the wall is less extensible than the inner or that the deposition of new material is limited to the inner layers of the wall.

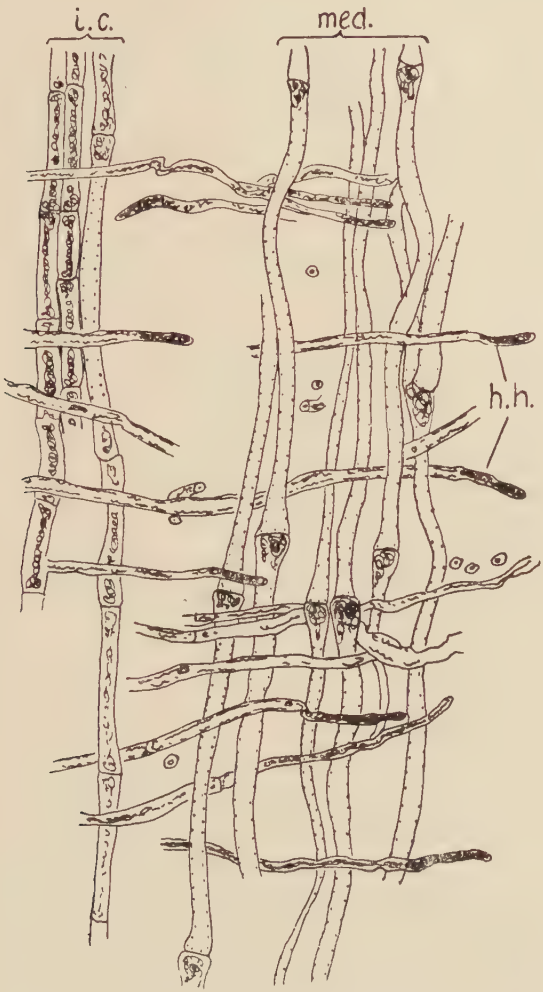
Cells 0·9 mm. in length were measured less than 2 in. behind the apex of receptacles only just 6 in. long, and cells 4 mm. long in receptacles 6 ft. long. This elongation of the medullary cells is the most extreme on record for any member of the Fucales, and indicates that the rapid growth in length of the receptacle is largely due to the great activity of the meristoderm.

Wille (1910) describes the elongation of the medullary cells, but does not describe anything like the degree of elongation here seen, the longest cells he figures having a length of only about ten times their diameter. He also mentions that the cells are slightly trumpet-shaped, but attributes this to a loss of turgor on handling, but in these extremely elongated cells I do not believe this to be the case. He also states that the nucleus is not always central in position, but has not seen the restriction of the protoplast to one end of the cell, always the distal, which is such a conspicuous feature of all the material I have handled (Text-fig. 10). The extreme elongation of the medullary cells of *H. lorea* is also mentioned by Delf (1939), who quotes Rees's measurements of cells 30 times as long as they are broad.

In the elongating receptacles a slight increase in the thickness of some of the walls can be seen as the distance from the apex increases. This is evident both in the inner cortex and in the medulla. In a few of the innermost layers of the cortex there may also be a certain amount of gelatinization of the outer layers of the cell walls, but this is never sufficient to cause very wide separation of the cells of this region.

Wille (1910) describes the medullary cells as sometimes appearing very thick walled, but believes this to be due to a post-mortem swelling of the inner layers of the walls as they come into contact with water on sectioning. This sometimes does occur, causing the innermost layers to swell so con-

siderably that the cell lumen is almost obscured, but this does not always account for the thickness of the cell walls. In a single receptacle 8 in. long, in the apical 2 in. the cells had thin walls as figured in Text-fig. 9, but as the



TEXT-FIG. 10. The medulla and the inner cortex of a receptacle 16 cm. long, in transverse section. *h.h.* = horizontal hyphae; *i.c.* = inner cortex; *med* = medulla.

distance from the apex increased the thickness of the walls also increased, until at the base of the receptacle the thickness of the walls exceeded the diameter of the cell lumen, so that the medullary cells here had the appearance of fulfilling a mechanical function.

In a similar manner, the thickness of the inner cortical cell walls increased

towards the base, but in these actively elongating receptacles the thickening of the walls was never very pronounced.

(*c*) *The mature receptacle*

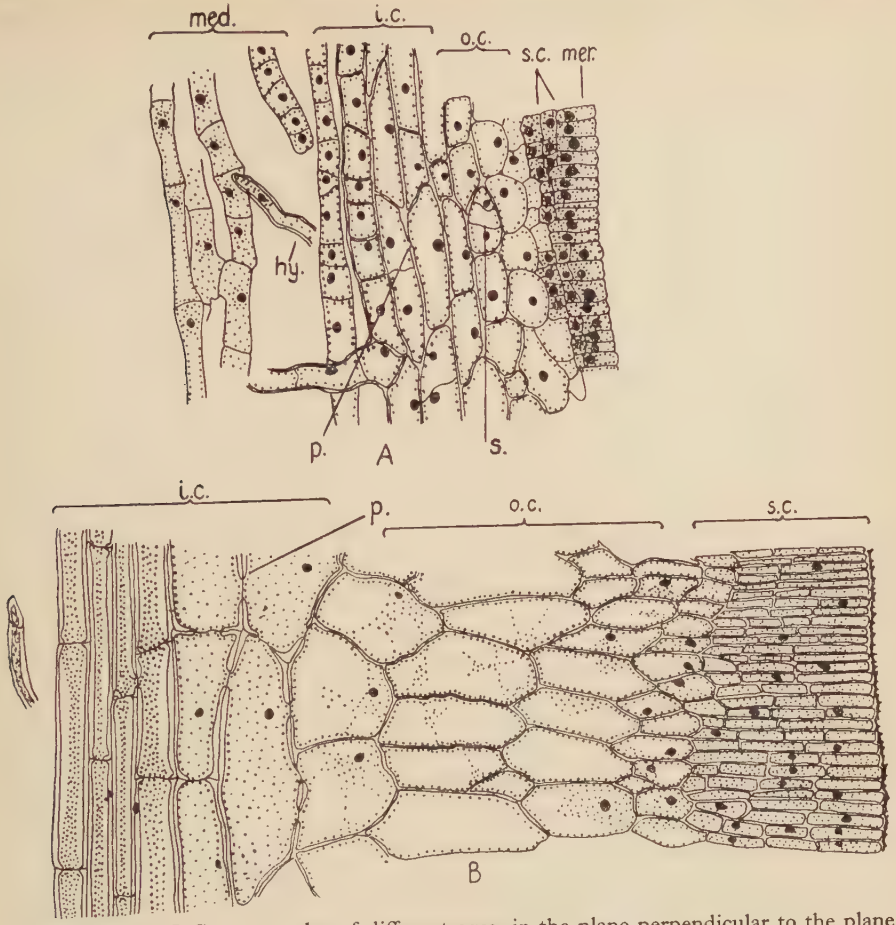
When most of the active elongation has taken place the apical groove and the apical cell disappear, as they do in the receptacles of *Fucus*. The apex narrows rapidly to a rounded peak which is bounded by a meristoderm and a cortex arranged in regular, radiating rows. Plants collected at the Lizard in June 1949 had already reached this stage, so that the further increase in length which they showed—from 4 ft. 6 in. to 6 ft.—between June and September must have been entirely due to the activity of the meristoderm, together with a general increase in cell size. The degree of cell enlargement seen in material from Clachan (Text-fig. 11) would alone be sufficient to account for an appreciable increase in length after the cessation of apical growth.

Owing, however, to the elongated form of the fronds, very few of the apices remain intact. It was only with difficulty that I could find any intact apices, many of the plants having the majority, if not all, of their tips worn down. The plants appear to possess a great capacity for wound healing in this region. The damaged apices were sealed by a compact and regular celled tissue produced by the regular segmentation of the neighbouring cortical cells. In some cases a very slight degree of elongation had taken place—about 1 mm.—by the repeated segmentations of this tissue. But this resulted only in the production of a solid tip, and I was unable to find any evidence of differentiation of new medullary cells. In one instance I found that this tissue had reproduced an apical groove complete with lining hairs, but I could find no new apical cell.

At this stage there is also an increase in the depth of the outer cortex as the result of periclinal divisions of the meristoderm, and finally the meristoderm gives rise to an outer tissue of very small cells arranged in regular rows and elongated in the direction perpendicular to the surface (Text-fig. 11). Thus an appearance is produced similar to that seen in the upper surface of the old button. Probably this tissue represents a final burst of activity of the meristoderm preceding the cessation of its activity. Osborn (1945) describes a similar small-celled tissue bounding the receptacles of *Hormosira Banksii*, and it is also visible in the older regions of the axis of *Halidrys siliquosa*.

The thickness of the inner cortical cell walls also increases with the age of the receptacle, and in some cases the thickening is laid down on the thin horizontal walls as well as on the longitudinal walls.

The medulla also changes in nature in the older receptacles. Often the extremely elongated medullary cells could be seen, but their cavities were usually obscured by the collapse of the walls in the middle regions. In many cases these cells were degenerating and in a few cases they could not be detected at all. Thus it seems that, if they have a conducting function, as suggested by Wille (1910), it can only be in the young stages before the



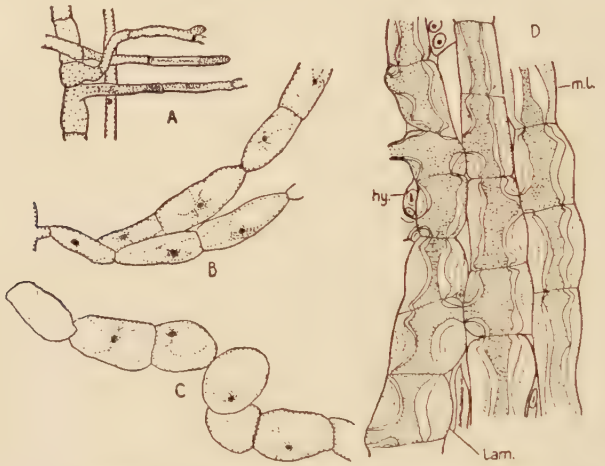
TEXT-FIG. 11. L.S. receptacles of different ages, in the plane perpendicular to the plane of flattening.

(A) L.S. immediately behind the apex of a young receptacle, showing the meristoderm (*mer*) and the small cells recently cut off from it (*s.c.*); the outer cortex (*o.c.*) of large thin-walled cells, roughly isodiametric or only slightly elongated, one cell showing transverse septations (*s*); and an inner cortex (*i.c.*) of more elongated cells, the walls of which are showing slight thickening and occasional pits (*p*).

(B) L.S. fully developed receptacle. There is a much broader outer region of small cells formed from the meristoderm. The size of the cells of the rest of the cortex, has greatly increased and so has the degree of thickening of their walls.

extreme elongation obscures the cavity. In these early stages the young receptacles are a paler colour than when mature, and are possibly not able to carry out sufficient photosynthesis to maintain themselves, and longitudinal conduction from the button may be necessary for their successful establishment. But in the mature receptacles there would appear to be no need for longitudinal conduction, since they are capable of assimilation along their whole length, and being shortlived there is no need of longitudinal conduction for storage purposes.

The appearance of the horizontal hyphae also changes. The individual cells begin to swell so that the hypha comes to consist of sausage-shaped cells (Text-fig. 12 B). In some cases the swelling proceeds still farther so that the cells eventually become spherical (Text-fig. 12 C). Thus in some cases where the horizontal hyphae were closely arranged the swelling results in the cells becoming closely packed together, giving an appearance of irregular blocks of parenchymatous tissue occupying the medulla. Wille (1910) describes the cells of the horizontal hyphae as having a slightly greater diameter at the centre than at the ends, but does not describe this very pronounced swelling.



TEXT-FIG. 12. A–C. Stages in the swelling of the horizontal hyphae. (Clachan material.)
(A) Horizontal hyphae from young receptacle.
(B) Hyphae from the mature receptacle showing early stages of swelling.
(C) Hypha from old plant showing fully swollen cells.
(D) L.S. of the inner cortex of an old receptacle. *m.l.* = middle lamella; *lam* = thickening of the wall, showing lamellations; *hy* = hyphae, cut transversely.

The number of longitudinal hyphae present also increases in the older plants. In the upper parts of the receptacles these are produced principally at the margins. Their numbers increase towards the basal regions until they form a complete cylinder running between the inner cortical cells. Finally, at the base, they extend into the medulla and form a solid core running between the medullary cells into the button.

The rounded basal region of the receptacle possesses from its first appearance a much more extensive cortex than the upper regions. The inner zone of regularly arranged cells is also broader than higher up. This greater depth may be correlated with the slower rate of elongation at the stage of formation of this region.

Whilst the changes just described are taking place, the oogonia and antheridia develop and the conceptacles become sexually mature. After this, except for further increase in the thickness of the cell walls, no further anatomical

developments take place, and, after the shedding of the gametes, the receptacles degenerate and become broken up.

Staining Reactions and Chemical Composition

The walls of the inner cortex of the receptacle give a very characteristic staining reaction with Gentian violet. Whereas the outer walls stain only very lightly with Gentian violet, these cell walls stain a very intense reddish-purple, a colour associated with the presence of mucilage. The region of the wall giving this reaction is a sharply defined layer in the central region of the wall, possibly the middle lamella, and it also stains with Ruthenium red. With Gentian violet the portion of the wall immediately surrounding the lumen stains only lightly or not at all. The cytoplasmic contents of the cells, in contrast, stain blue. In their staining reactions these cortical cells thus resemble the cortical cells of the stipe of *Fucus vesiculosus* (Moss, 1948), except that in *F. vesiculosus* the central deeply staining region seems to be rather broader than in *H. lorea.*

At the inner margin of the cortex where gelatinization has somewhat separated the cells, each cell is surrounded by a narrow, non-staining region; a sharply defined, deeply staining region; and finally, a wider lightly staining region separating the deeply staining regions. In the cortex of the button, where the gelatinization is more pronounced, a similar reaction is seen.

In the inner cortical cells where the thickening is very marked, on very deeply staining with Gentian violet, narrow bands, deeply staining and of a granular appearance, can be seen alternating with wider, clear, and only very lightly staining regions (Text-fig. 12 D).

The application of iodine followed by concentrated sulphuric acid shows that cellulose is widely distributed in the plants. In the young sporelings 2–3 mm. high a very pronounced reaction is obtained in the walls of the medullary cells, cortical cells, and meristoderm cells. In the cortex and meristoderm, only a thin layer immediately surrounding the protoplast gives the cellulose reaction, but in the medulla the cellulose appears to be present throughout the wall. A reaction is only obtained with difficulty in the walls of the hyphae, but is quite clear at their tips. As the sporeling flattens out to form the button, the cellulose reaction is particularly marked in the cortical cells at the rim of the button, a region which can be felt to be firmer than the rest of the sporeling. By the time that the flattened, solid button is produced, a cellulose reaction is given by the walls of all the cells present.

Cellulose is also widely distributed in the receptacles. As in the button, in the meristoderm and cortex the cellulose is only present in the layer of the wall lining the protoplast, the middle regions of the walls giving no reaction. It is also present in the walls of the medullary cells and in both the longitudinal and the horizontal hyphae.

An interesting feature is the cellulose reaction of the thickened walls of the inner cortical cells. Here narrow bands of cellulose alternate with wider zones of the wall containing no cellulose. In one extremely thick wall as

many as fourteen bands of cellulose were counted. This banding makes an interesting comparison with the staining reaction of these cells with Gentian violet, and may perhaps be compared with the concentric lamellae seen in the walls of the medullary cells of *Fucus* (Moss, 1948; Naylor and Russell-Wells, 1934). The occurrence of a cellulose layer lining the protoplast is quite a usual feature of the Phaeophyceae (Naylor and Russell-Wells, 1934).

Moss (unpub.) has shown that alginic acid is a major chemical constituent of the receptacles of *H. lorea*. As no microchemical test is known for this substance, attempts were made to determine its distribution by extracting it from sections of the receptacle. Sections of the fresh material, slightly air-dried, were soaked overnight in 0·2 N sulphuric acid, and then transferred for a similar period to sodium carbonate solution. This treatment is the recognized method for the quantitative extraction of alginic acid, which is extracted in the form of the sodium salt, yielding a brown viscous liquid.

The extreme difficulty of handling the sections after this treatment shows that some component, either of the cell wall or of the intercellular mucilage, has been removed. It was only with extreme difficulty that sections so treated could be mounted on a slide, and putting on a coverslip usually caused the whole section to disintegrate. It was found, as Moss (1948) found in *Fucus vesiculosus*, that the central tissues in particular fell apart owing to the removal of the intercellular matrix. In the medulla there had accumulated large, amorphous masses, brown in colour, which were possibly the extracted sodium alginate. A further point of interest was revealed on staining with Gentian violet; namely, that little was left of the lamellate thickening of the walls of the inner cortical cells, and that in the thinner portions of the sections these had been completely removed, leaving only the deeply staining middle lamellae. It thus appears that alginic acid, as well as cellulose, is a constituent of these walls.

That these changes were in no way due to the swelling and softening effect of the sulphuric acid alone was checked by examining the sections after the acid stage of the treatment. The cell walls are very resistant to swelling with sulphuric acid, and after several days' immersion in the acid, the cell walls remain firm and hardly swollen. The sections are still firm and easy to handle, and after mounting, considerable pressure on the coverglass will not cause them to disintegrate. The thickening of the walls of the inner cortex is still firm and unaffected by this treatment.

In the medullary cells of the stipe of *F. vesiculosus* Moss (1948) found that the alginic acid occurred separating the cellulose lamellae. Thus both in the cellulose bands, and in the presence of alginic acid in their walls, these inner cortical cells of *H. lorea* resemble the medullary cells of the stipe of *F. vesiculosus*.

Comparisons of Plants from Different Habitats

Receptacles of plants from different types of habitat were compared and showed considerable divergence from each other, both in the external form

of their receptacles and in the details of their anatomical structure. The three principal localities from which plants were collected were Clachan Sound, Argyllshire; Trearddur Bay, Holy Island; and Lizard Point, Cornwall. Plants were collected from these three habitats at different times of the year, and comparisons made at as many stages of development of the receptacles as possible.

Clachan Sound is a narrow tidal channel separating Seil Island from the mainland, through which there is normally a strong tidal current. Here *H. lorea* grows very near, often below, low-water mark, with *Laminaria digitata*. At the very lowest spring tides in April 1949 the majority of the plants were not uncovered at all, and a small number were exposed for about one hour. Thus the amount of intertidal exposure that these plants have to withstand is very slight.

Trearddur Bay[1] 'is a few miles to the south of Holyhead and faces the open sea. The *H. lorea* here occurs in a wide belt about 8 ft. wide, just above the *Laminarias*, and is on a sloping face mixed with *Laurencia* and *Gigartina*. It is on an exposed headland facing south to south-west, and the zone ends abruptly as one passes into the more sheltered bay.' The plants here are at

TABLE I

Summary of the Receptacles used in the Comparative Investigation

Locality.	Stage I (elongating).	Stage II (maturing).	Stage III (mature).	Stage IV (degenerating).
Clachan Sound, Argyllshire.	5 ft. long. 0·2 in. broad. 1 ft. sterile. 15/5/49.		6 ft. 6 in. long. 0·5 in. broad. 4 in. sterile. 24/10/49.	2 ft. long. 0·4 in. broad. 15/5/49.
Trearddur Bay, Anglesey.	8 in. long. 0·2 in. broad. 1·5 in. sterile. 24/4/48.		2 ft. long. 0·3 in. broad. 2 in. sterile. 23/10/49.	
Lizard Point, Cornwall.		4 ft. long. 0·2 in. broad. 6 in. sterile. 14/6/49.	6 ft. long. 0·5 in. broad. 4 in. sterile. 26/9/49.	

a slightly higher level than at Clachan Sound, and the degree of intertidal exposure is correspondingly greater, varying up to 2–3 hours at the low spring tides.

At Lizard Point the *H. lorea* covers a wide zone of rocks, growing both in completely submerged positions and also in a rather scattered manner in the intertidal region up to levels on the shore where, at the low spring tides, they were exposed for all the 4 hours I spent collecting.

It is not easy to make an estimate of the relative degree of exposure to wave action to which the plants from these three localities are subjected, but the

[1] I am indebted to Dr. M. T. Martin of Bangor for the collection of the plants from this locality, and also for this description of the shore.

two latter habitats where the rocks face the open sea must at times be more exposed than the first, where the Sound is separated from the sea by Seil Island.

Comparisons of these three habitats show that there is a considerable range in the level at which the *H. lorea* occurs on the shore. Gibb (1937) found that at Port St. Mary the zone extended from 1 ft. above L.W.O.S.T. to $3\frac{1}{2}$ ft. above L.W.N. She found no plants growing in completely submerged situations, such as seen at Clachan and at Lizard Point. Possibly the upper limit is conditioned by the extent of the 'spray zone' which will be broader in the more exposed situations.

The length of the mature receptacles varied considerably in the different localities. In 1949 2 ft. was the average length reached by the receptacles at Trearddur Bay; 6 ft. by those at Lizard Point; and 7 ft. by those at Clachan Sound (Table I).

At both Lizard Point and at Trearddur Bay the length of the receptacles varied with the amount of submergence, the lower plants possessing the longer receptacles. In 1949, at Trearddur Bay, the plants at the higher levels were extremely short, not more than 1 ft. in length, and at Lizard Point the plants at the upper limit of the zone were about 1 ft. shorter than the completely submerged ones. This agrees with Gibb's (1937) observations at Port St. Mary, where she found that towards the lower limits of the *H. lorea* zone the average length was about 100 cm., whereas at the upper limits the average was only half that amount.

For comparisons of the anatomical structure, it was found most convenient to make comparisons between receptacles showing the same degree of development of the conceptacles, since the very great variation in the lengths of the mature receptacles made it impossible to take the length as a basis for comparison.

One point which becomes evident from this manner of separation into developmental stages is that the gametangia do not begin to develop until the initial rapid elongation of the receptacle has ceased. The 5 ft. long receptacles collected from Clachan Sound in April 1949 showed the same degree of development of the conceptacles as did the 8 in. long ones collected at Trearddur Bay, also in April.

The chief anatomical features influenced by the environmental conditions are longitudinal hyphal development, the development of the cortex, and the elongation of the medullary cells.

Longitudinal hyphal development is much more pronounced in the receptacles from Trearddur Bay and Lizard Point, where exposure to wave action is greater, than in receptacles from Clachan, where the exposure is less. A similar greater development of hyphae in plants from more exposed habitats was seen by Moss (1948) in *F. vesiculosus*.

The thick-walled region of the inner cortex is more extensive and the individual cells thicker walled in the receptacles from Trearddur Bay than in those from Clachan Sound. Those from the Lizard are intermediate in this

respect. This feature seems to be related to the level on the shore at which the plants grow, those at the higher levels having a broader and thicker walled inner cortex than those at the lower levels. This suggests that the thick-walled cells may play some part in water retention during the ebb, as suggested by Henckel (1912) in *Pelvetia canaliculata.*

The degree of extension of the medullary cells is very much greater in the longer receptacles, individual cells measuring 4 mm. in length being found in the receptacles from Clachan Sound, compared with a length of 1 mm. in the shorter receptacles. This indicates that surface growth is more active in these longer receptacles, and this feature is no doubt related to conditions of the environment, possibly nutritional as well as level on the shore and degree of wave action.

Thus it can be seen that, although the same general course of development is followed in all cases, conditions of the environment influence the details of the anatomical structure.

Discussion

Comparisons with other Fucales

(*a*) *General morphology.* On account of the peculiar form of its thallus, *H. lorea* is usually classified by itself in the group Loriformes (Oltmanns, 1889; de Toni, 1895; Grüber, 1898; Schmidt, 1938; Fritsch, 1945).

The external form of the thallus shows few points of similarity to the other members of the Fucales. It resembles the Fucaceae in the possession of a radially organized sporeling, which later gives rise to a flattened thallus branching in one plane. The radial organization is retained longer here than is usual in the Fucaceae, and whereas in the Fucaceae this change in symmetry accompanies a change from a three-sided to a four-sided apical cell, in *H. lorea* growth is carried out throughout its life-history by the same three-sided apical cell, as is the case in the Cystoseiro-Sargassaceae.

The production of a bilateral—and in some cases flattened—thallus from a three-sided apical cell is also seen in the Cystoseiro-Sargassaceae. In the Cystoseiraceae *Halidrys siliquosa* and *Bifurcaria tuberculosa* have a bilateral organization, and *Carpoglossum* and *Platythalia* are both bilateral and flattened. The closest superficial resemblance is to *Carpoglossum*, where the receptacles are also elongated and strap-like. *H. lorea*, however, differs from all these types in its dichotomous branching, whilst the Cystoseiraceae all possess monopodial growth. The branching of *B. tuberculata*, although apparently dichotomous, is reported by Grüber (1898) to be monopodial and bilateral. The radial organization of the Cystoseiraceae is believed to be secondary in origin, the flattened forms being the more primitive. In *C. abrotanifolia* (Valiante, 1883), for example, at an early stage the young sporeling flattens out and resembles closely a young *Fucus*; this original axis does not develop very far, and growth is continued by another apical cell which arises near the base of the sporeling and gives the characteristic radial organization of the adult plant. Amongst the Sargassaceae, both bilateral and

radial forms occur within the genus *Sargassum*, and the genus *Carpohyllum* is both bilateral and flattened. As in the Cystoseiraceae, these flattened and bilateral forms are considered to be the least specialized members of the group.

Fritsch (1945*a*) interprets the receptacles of *H. lorea* as 'one fork of a dichotomy, the other limb of which remains arrested'. The button he interprets as the primary axis, and compares its development with the primary axis of *C. abrotanifolia*. But such an interpretation involves the sympodial or adventitious development of the receptacles from an apical cell distinct from that which produces the button, and there is no evidence that this is the case.

(*b*) *Structure and development.* Throughout the Fucales the primary tissues of the thallus are built up from two sources: from the segmentation of the apical cell, and by the activity of the meristoderm. As the result of the very regular segmentation at the apex, the medulla and the innermost elements of the cortex come to be arranged in very regular vertical series, whilst the other tissue do not show such regular arrangement. As the result of the surface growth due to the horizontal divisions of the meristoderm, the inner cells, which soon lose their power of division, become stretched in the direction of the longitudinal axis. Periclinal divisions of the meristoderm add to the depth of the cortex. The ultimate structure of the inner tissues appears to depend on the amount of submergence of the plant. In the intertidal genera, including *H. lorea*, there is abundant gelatinization of the longitudinal walls of the medullary cells, so that the cells become separated into longitudinally running filaments supported in a mucilaginous matrix. In the submerged genera, on the other hand, there is no gelatinization, and a compact parenchymatous structure is retained throughout the thallus.

This gelatinization of the middle layers of the walls of the medullary cells is seen in those intertidal Fucaceae which have been investigated—i.e. in *Pelvetia* (Hansteen, 1892; Henckel, 1912); in *Fucus* (Oltmanns, 1889); in *Ascophyllum* (Oltmanns, 1889; Reinke, 1876 'Ozothalia nodosa'); and in *Xiphophora* (Heine, 1932; Reinke, 1876 'Fucus chondrophyllus')—and also in *Hormosira* (Osborn, 1945), in *Notheia* (Barton, 1891), and in *Durvillea* (Naylor, 1949). The compact, parenchymatous type of organization is seen in those Cystoseiraceae—i.e. *Cystoseira* (Reinke, 1876; Berthold, 1882; Dodel-Port, 1885); in *Bifurcaria* (Rees, 1932); and in *Halidrys* (Fritsch, 1945)—and in those Sargassaceae—i.e. in *Sargassum* (Hansteen, 1892); and in *Turbinaria* (Barton, 1891)—which have been investigated. It is also reported by Reinke (1876) in some of the submerged Fucaceae—in *Marginariella boryana* ('Sargassum Boryanum') and in *Axillaria constricta* ('Carpoglossum constrictum')—and I have seen it in those submerged genera of the Fucaceae which I have examined—i.e. in *Scytothalia dorycarpa* and in *Phyllospora comosa*.

In some of the intertidal genera, but not in *H. lorea*, this gelatinization extends into the cortex as well as the medulla. Examination showed that this gelatinization extended to nearer the meristoderm in plants from the higher

levels on the shore than in those from near low-water mark. The cortex of *H. lorea*, however, shows much greater similarity to the inner cortex of the completely submerged forms, where there is also no gelatinization. *Halidrys siliquosa*, which has been the most completely investigated of these forms (Fritsch, 1945), shows very striking similarities in the thick, lamellate walls of the inner cortex. Such thick lamellate walls are not a usual feature of the intertidal Fucaceae which have been investigated, but I have seen them in various submerged Fucaceae—in *Scytothalia dorycarpa* and in *Phyllospora comosa*. They are also figured in *Hormosira Banksii* by Osborn (1948). In the axis of *H. siliquosa* this cylinder of thick-walled cells forms a very conspicuous feature—attention was drawn to it by Reinke (1876)—and probably has a mechanical function. It is interesting that in the intertidal genera the comparable region is occupied by the longitudinally running hyphae.

It thus seems that the nature of the cell wall throughout the cortex is fundamentally the same in all the groups of the Fucales. The development of mucilage in the cortex is dependent on the amount of submergence of the plant, and does not constitute a feature of systematic importance as can be seen from the fact that within the group of the Fucaceae there are found genera in which mucilage production occurs and others where it is absent.

(*c*) *Hyphal development.* The intertidal genera with their extensive gelatinization are usually characterized by much more abundant production of longitudinally running, mechanical hyphae than the compact, parenchymatous forms, where they are only produced in the basal regions, and places where extra strength is required. In the elongated receptacles of *H. lorea* only relatively few longitudinal hyphae are produced except in the basal regions. As in *F. vesiculosus* (Moss, 1948), more hyphae are produced in plants from exposed habitats than in those from more sheltered ones.

Production of short, horizontally growing hyphae from the inner cortical cells is quite an unusual feature in the Fucales, although common in the Laminariales. It makes an interesting comparison with *Durvillea antarctica*, which, both in the form of the adult thallus and in its exposed habitat near the lower limit of the littoral region, shows further points of similarity to *H. lorea*. This production of horizontal hyphae may be correlated with the exposed nature of the habitat, or with the formation of the central air chambers which help to bring the elongated fronds to the surface of the water.

(*d*) *Alginic acid.* The amount of alginic acid present in the fronds of *H. lorea* is a further point of interest, as this is a substance sometimes correlated with flexibility (Moss, 1948), and the elongated receptacles of *H. lorea* are particularly flexible structures. Moss (1948) found that in *F. vesiculosus* the percentage alginic acid was highest in the tips and decreased towards the base of the plant. She also found that in plants from exposed habitats the percentage alginic acid in the tips of mature plants was always greater than in the tips of plants from less exposed habitats. The amount of alginic acid present in the tips of plants from Cullipool was found to be 20·7 per cent. of

the dry weight, compared with 16·6 per cent. of the dry weight in the tips of plants from the less exposed habitat of Loch Melfort.

The percentage of alginic acid present in the receptacles of *H. lorea* was found by Moss (unpub.) to be very high, 28 per cent. of the dry weight being reported in the oldest plants examined. The percentage alginic acid is also reported to be very high in *Durvillea antarctica* (= *D. utilis*, Marini-Bettolo, 1948), where it is estimated as 67 per cent. of the total polysaccharide. It thus seems that alginic acid is a major constituent of these long and flexible fucoids of exposed habitats.

Possible affinities

In the possession of the distinctive three-sided apical cell *H. lorea* resembles the Cystoseiraceae and the Sargassaceae, and apart from *H. lorea* this feature is not seen outside these two groups. The dichotomous branching, the formation of mucilage in the medulla, and the horizontal hyphae are the chief features which separate *H. lorea* from the Cystoseiraceae, and it is possibly with the flattened members of this group that its affinities are to be found.

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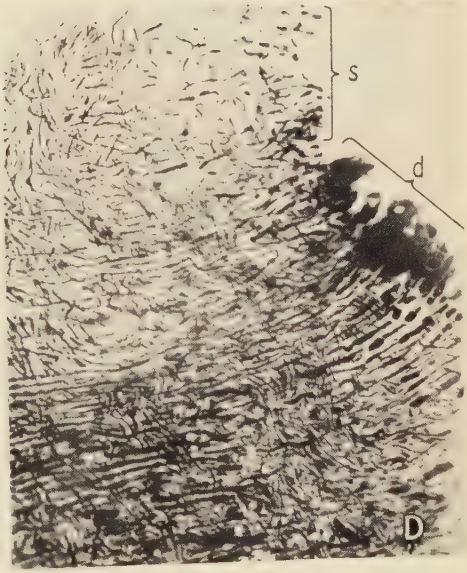

Stages in the development of the button of *H. lorea*.

A. 1, 2, 3, and 4, young pear-shaped, fluid-filled sporelings. 5, the beginning of the flattening, the plant still soft and fluid filled, and the apical groove clearly visible. 6, 7, and 8, flattening well advanced, giving a tough, leathery button.

B. L.S. young sporeling, 3 mm. long, showing the central elongated medullary filaments (*med*) and the hyphae (*hy*).

C. L.S. stipe of a well-developed button showing the numerous interweaving hyphae and the filaments of medullary cells (*med*). *b* = point of branching of a hypha.

D. L.S. of the transition region between stipe and disc of a well-developed button, showing the numerous hyphae growing downwards and outwards and interweaving to form the compact disc. The terminal segmentation of the hyphae to form a cortex can be seen at the upper surface of the disc (*d*).

M. NAYLOR

Two Natural *Agropyron* Hybrids occurring in the British Isles

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With Plate XXVI and two Figures in the Text

ABSTRACT

This paper contains comparative descriptions of two natural Agropyron hybrids found in the British Isles, *A. pungens*×*junceum* and *A. repens*×*junceum*. It records the flowering periods of the parents and of *Elymus arenarius*. It also summarizes the various intergeneric crosses possible within the tribe Hordeae.

1. INTRODUCTION

CONSIDERING the large number of wild species in the Hordeae, the number of natural hybrids recorded is small (see Appendix). Those which have been studied in any detail, however, have given valuable information bearing on the taxonomy and the evolution of the tribe. Many of the species of the earlier taxonomists have been found to be interspecific natural hybrids, or possibly even mixtures of two natural hybrids, as may be the case for '*Elymus Macounii*' (Vasey) (Stebbins, Valencia, and Valencia, 1946*a*). Some intergeneric natural hybrids show by their ease of production in nature that the species involved are much more closely related than their present classification suggests, and this is supported by cytological investigations. Thus Stebbins and his co-workers (1946*a*) have shown that in a *Sitanion–Elymus* hybrid the chromosomes of the two parent species pair very regularly at meiosis and suggest that from a cytogenetic point of view the two genera cannot be considered distinct.

This paper contains descriptions of two natural *Agropyron* hybrids which occur in the British Isles and is a preliminary to an account of their chromosome cytology. Both these hybrids have been described from other localities. Simonet (1934) discusses *A. pungens*×*junceum* from the French coast, while Östergren (1940) has examined *A. repens*×*junceum* from southern Sweden. This paper adds further observations to those made by these two authors.

2. *AGROPYRON PUNGENS*×*JUNCEUM* (Plate XXVI)

On many sandy coasts of the British Isles *Agropyron junceum* (L.) Beauv. and *Agropyron pungens* (Pers.) Roem. and S. grow in close proximity. The

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former species occurs on the fore-dune, and the latter species on the fixed dune, often no more than 10 yards inland. The two species are thus ideally situated for interspecific hybridization, particularly as they occur as ecological dominants in very large numbers. On the seaward margin of the *A. pungens* area there is commonly found a form which agrees with the description of *Agropyron acutum* Roemer and Schultes. *A. acutum* is intermediate in appearance between *A. junceum* and *A. pungens*, and Duval-Jouve (1875) suggested that it was a natural hybrid between the two species. That this was true was shown by Simonet (1934) working on French material, who found that the chromosome number of *A. acutum* is 35, which is the sum of the haploid complements of *A. pungens* ($2n = 42$, Peto, 1930) and *A. junceum* ($2n = 28$, Peto, 1930).

In Table I are compared the average measurements obtained when 10 plants of each of *A. pungens*, *A. junceum*, and their natural hybrid were scored

TABLE I

Morphological Comparison of A. pungens, A. junceum, *and their Natural Hybrid*

Character.	*A. junceum.*	*A. junceum* × *pungens.*	*A. pungens.*	Remarks on hybrid.
Habit	Decumbent. Culm 40–50 cm. horizontal to vertical. Rhizomatous	Medium tall. Culm 70–80 cm. vertical or slightly drooping. Rhizomatous	Tall grass. Culm 90–115 cm. vertical. Rhizomatous	Intermediate
Habitat	Fore-dune	Fixed dune	Fixed dune	*pungens* dominates
Flowering time	Early	Later	Later	*pungens* dominates
Plant colour	Glaucous, green	Glaucous	Glaucous	*pungens* dominates
Leaf	Green and glabrous below. Glaucous and prominently veined above	Glaucous and prominently veined above	Glaucous and prominently veined above	*pungens* dominates
Spike length	16·5 cm. (S.D. 1·86 cm.)	15·4 cm. (S.D. 2·00 cm.)	11·5 cm. (S.D. 1·75 cm.)	*junceum* dominates
Spikelets per spike	7–11	12–20	15–25	Intermediate
Internode length	1·8 cm. (S.D. 0·17 cm.)	0·9 cm. (S.D. 0·11 cm.)	0·43 cm. (S.D. 0·19 cm.)	Intermediate
Rhachis margin	Smooth	Smooth	Serrate	*junceum* dominates
Disarticulation of rhachis	Readily, just above each node	None	None	*pungens* dominates
Spikelet length	2·2 cm. (S.D. 0·11 cm.)	1·7 cm. (S.D. 0·15 cm.)	1·7 cm. (S.D. 0·18 cm.)	*pungens* dominates
Flowers per spikelet	4–5	5–6	5–9	Intermediate
Length glume / Length spikelet	0·66–0·75	0·5–0·66	0·5	Intermediate
Apex of lemma	Mucronate or obtuse	Mucronate or obtuse	Emarginate, mucronate, acuminate, or awned	*junceum* dominates

for 14 characters. It can be seen that *A. pungens* dominates in 6 characters, *A. junceum* in 3, and that 5 characters are intermediate between the two parents.

The chromosome number of the hybrid was found to be $2n = 35$ as expected. The complications of chromosome pairing in this pentaploid, which will be described in another paper, almost invariably led to the production of empty pollen-grains and the hybrid is quite sterile. The pollen of four hybrids was tested by staining with iodine, and samples of 1,000 grains from each plant showed only 6, 1, 0, and 1 good grains. Many hundreds of spikes were examined, but no seeds were found.

3. *Agropyron repens*×*junceum* (Plate XXVI)

This hybrid has been collected from five localities in southern Sweden by Östergren (1940), and British botanists have often collected it on the British coasts where farmland abuts on sand-dune and the two species grow in proximity. However, if *A. pungens* is also present in the area it would be very difficult to distinguish this hybrid from the one previously described. The hybrids have one parent in common, *A. pungens* and *A. repens* are quite similar morphologically, and the chromosome number would be 35 in both cases.

As *A. pungens* occurs no farther north than about Dublin in Eire, it was decided to collect *A. repens*×*junceum* from Northern Ireland, and abundant material was found at Portavogie on the County Down coast. Here farmland and beach meet, and on the consolidated sand at the back of the beach *A. repens* grows amongst *Festuca rubra*, *Ammophila arenaria*, *Raphanus maritima*, *Rumex crispa*, *Matricaria inodora*, *Galium aparine*, and *Sonchus arvensis*. *A. junceum* is found on the seaward margin of this association and the hybrids with *A. repens* occur here.

The morphology of *A. repens*×*junceum* is compared with that of its two parents in Table II. Ten plants of each type were measured. Of the 14 characters studied *A. junceum* dominates in 4, *A. repens* dominates in 1, 5 are intermediate, 2 show an increase on either parent species, and 1 a decrease.

Although it was not possible to examine pollen of this hybrid or to search for seeds, it would be quite safe to say that it is quite sterile as meiosis shows many irregularities (Godley, 1947).

4. General Characteristics of the Hybrids

In both hybrids, although *A. pungens* and *A. repens* have contributed half as many chromosomes again as *A. junceum*, there is no marked predominance of the characters of the hexaploid. Indeed, in *A. repens*×*junceum* only one character of the hexaploid definitely dominates.

When the dominance relations of the characters studied in the two hybrids are compared the following general points emerge.

(i) Those characters such as habit, height of culm, internode length, and

TABLE II

Morphological Comparison of A. repens, A. junceum, *and their Natural Hybrid*

Character.	*A. junceum.*	*A. repens*×*junceum.*	*A. repens.*	Remarks. on hybrid.
Habit	Decumbent. Culm 40–50 cm. horizontal to vertical. Rhizomatous	Semi-decumbent. Plants 30–50 cm. high. Culm not measured. Rhizomatous	Erect. Culm 70–90 cm. Rhizomatous	Intermediate
Habitat	Fore-dune	Fore and fixed dune	Inland and fixed dune	Intermediate
Flowering time	Early	Not observed	Early–late	—
Plant colour	Glaucous, green	Glaucous, green	Green or glaucous	*junceum* dominates or intermediate
Leaf	Green and glabrous below, prominently veined above	Green and glabrous below, prominently veined above	Green or glaucous. Thin veins both surfaces. Upper surface pubescent scabrid	*junceum* dominates
Spike length	16·1 cm. (S.D. 1·63 cm.)	20·1 cm. (S.D. 2·04 cm.)	12·6 cm. (S.D. 2·74 cm.)	Increase
Spikelets per spike	7–11	13–21	18–28	Intermediate
Internode length	1·7 cm. (S.D. 0·15 cm.)	1·25 cm. (S.D. 0·206 cm.)	0·42 cm. (S.D. 0.01 cm.)	Intermediate
Rhachis margin	Smooth	Smooth	Serrate	*junceum* dominates
Disarticulation of rhachis	Readily, just above each node	None	None	*repens* dominates
Spikelet length	2·3 cm. (S.D. 0·12 cm.)	1·8 cm. (S.D. 0·15 cm.)	1·24 cm. (S.D. 0·22 cm.)	Intermediate
Flowers per spikelet	4–5	2–3	3–5	Decrease
$\frac{\text{Length glume}}{\text{Length spikelet}}$	0·63–0·76	0·8–0·9	0·5–0·8	Increase
Apex of lemma	Mucronate or obtuse	Mucronate	Mucronate, acuminate, or awned	*junceum* dominates

number of spikelets per spike, which are probably under the control of many genes, are roughly intermediate in the hybrids.

(ii) Spike length shows an increase on either parent in *A. repens*×*junceum*. This effect is not a chance variation as it was also found in the Swedish hybrids studied by Östergren. The measurements now available enable us to test the suggestion made by Östergren that this increase is due to a favourable combination of two characters, internode length and internode number (i.e. spikelets per spike less one).

A. repens has a large number of short internodes, while *A. junceum* has a smaller number of longer internodes. In the hybrids both these characters are almost intermediate, but each has a slight bias towards the larger parental character. The product of these two components gives the heterotic effect in the hybrid.

In *A. pungens*×*junceum* both characters are again roughly intermediate, but although the number of internodes shows a bias towards the larger parent, the length of internodes shows a bias towards the shorter parent, and no heterotic effect is obtained in the hybrid.

iii. Plant colour in both hybrids is glaucous. This is to be expected as it has been shown that in *A. repens* glaucous plant colour is dominant to green (Godley, 1947) and dominance of glaucous is also found in *Triticum* (Watkins, 1927) and in *Secale* (Tschermak, 1906).

iv. Mucronate or obtuse apex to the lemma is dominant to awned. This again is to be expected as in *A. pungens* awnless was found to be dominant to awned (Godley, 1947) and in *Triticum vulgare* crosses between awnless or tip-awned and awned plants give hybrids which are close to the former types (Watkins and Ellerton, 1940).

v. Tough rhachis dominates brittle rhachis. This result is of interest as Love and Craig (1919) record the dominance of the brittle rhachis of *Triticum dicoccoides* in crosses of this species with *T. durum* and *T. vulgare*. Again Stebbins et al. (1946*a*) list three hybrids with fragmentation of rhachis intermediate between tough and brittle rhachis parents.

These three different results in the Hordeae cannot be explained as a result of different gene doses in the different crosses. The hybrids described by Stebbins et al. are between tetraploid species and are all constant in showing intermediate rhachis fragmentation, but in the *Triticum* cross the brittle rhachis of the tetraploid is dominant, whereas in the *Agropyron* cross the brittle rhachis of the tetraploid is recessive.

vi. Smooth rhachis margin dominates serrate margin.

vii. Leaf veining approaches the more prominently veined parent. (This is a superficial observation as no sections of the leaves were cut.)

viii. In *A. repens*×*junceum* the number of flowers per spikelet shows a decrease when compared with either parent. This depressing effect of the hybrid genotype on a character is also seen in *Geum intermedium* studied by Marsden-Jones (1930) which has no glands above the achene though these are present in both its parents.

ix. As a direct result of the reduction in flower number in *A. repens*×*junceum* this hybrid has a larger ratio of glume length to spikelet length than either parent.

5. Flowering Periods (Fig. 1)

It would be expected that the flowering periods of the species concerned in natural hybridism would overlap, but this was not so for *A. pungens* or *A. junceum* at Heacham in 1946 and 1947. At Heacham in both years *A. junceum* was in fruit before either *A. pungens* or *A. pungens*×*junceum* had commenced flowering. *A. pungens* overlapped with the hybrid in both years so that a back cross was possible.

As hybridization has definitely occurred in this area it must be concluded to be sporadic, and dependent on the influence of weather which causes

occasional overlapping in the flowering time. In the excellent summer of 1947 the flowering times were from 2 to 3 weeks earlier in all species as compared with 1946. A particular combination of weather conditions could conceivably cause an overlapping of flowering periods. It was noted in 1946 that on July 22 *A. pungens* had ripe anthers but that they were unextruded probably owing to the rainy weather. It seemed that only one hot day was needed to cause anthesis and bring the flowering time closer to that of *A. junceum*.

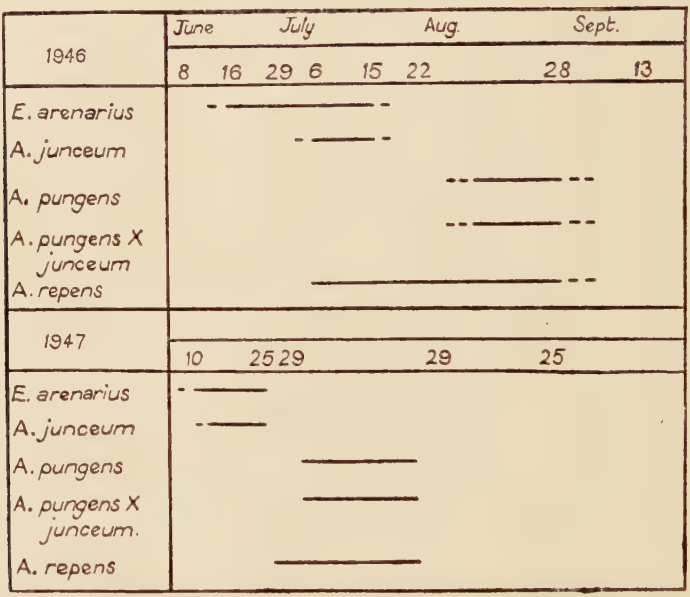

FIG. 1. Flowering periods (1946, 1947) of species and hybrids studied. *A. repens* at Cambridge, remainder at Heacham, Norfolk.

Plants of *A. pungens* grown in the warm house at Cambridge during the winter flowered 2–3 weeks earlier than those in nature, and at the same time as *A. junceum*. A mild winter combined with a warm summer may be the conditions necessary to cause overlap of the flowering times. Further observations may show, however, that *A. junceum* always flowers earlier than *A. pungens* and that hybridism is the result of crossing between occasional abnormally late individuals.

A. repens growing at Cambridge overlapped in both years with *A. junceum* recorded at Heacham.

A. junceum and *Elymus arenarius* had almost identical flowering periods in both years, and as these species grow intermixed they are ideally situated for natural crossing to occur. A possible *Elymus–Agropyron* hybrid has been recorded by Hubbard (1936) from Snettisham, which is quite close to the area under investigation. This author suggests that one parent was certainly

Elymus arenarius and the other possibly *A. pungens*. On the evidence of flowering times *A. junceum* seems the more probable *Agropyron* parent.

Hybridism between *E. arenarius* and *A. junceum* seems possible but is apparently at least very rare. The rarity may be due to the breeding systems of the two species. *A. junceum* is capable of self-fertilization, and this is also true for *E. arenarius*, for 123 flowers gave 53 seeds when an inflorescence was bagged. Thus the chances of natural crossing are considerably reduced.

An attempt to synthesize this hybrid in 1946 was unsuccessful. Fourteen florets of *A. junceum* set no seed when pollinated by *E. arenarius*.

6. The Hordeae Comparium

The genus *Agropyron* is one of eight genera in the tribe Hordeae which form intergeneric hybrids with facility, and it has been pointed out by Clausen, Keck, and Hiesey (1945) that these genera are all members of the same comparium.

The intergeneric crosses so far made are illustrated in Fig. 2, using information obtained from the summaries of Aase (1935, 1946) and from the list of hybrids in the Appendix to this paper. The eight genera are connected either directly or indirectly with each other by the various intergeneric hybrids which have been made.

The genus *Agropyron* occupies a very interesting position. It serves as a connecting link between the sub-tribe Elymineae and the sub-tribe Triticineae to which it belongs. (I use the classification of Hubbard in Hutchinson, 1934.)

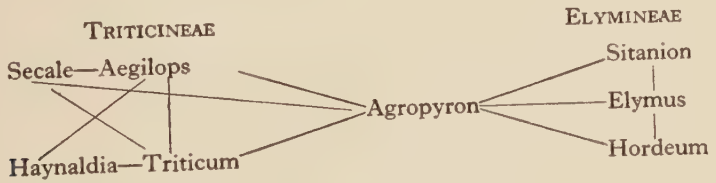

Fig. 2. The Hordeae Comparium

A general rule may be suggested on the evidence available. *No hybrids may be produced by normal methods between the Triticineae and the Elymineae except via the genus* Agropyron. The exceptional crosses prove this rule. Thus Soulier (1945) has combined chromosomes from *Triticum* and *Elymus* in the same plant, but only by using *Agropyron* as a bridge genus and forming a tri-generic hybrid. Direct crosses between genera of the two sub-tribes (excluding *Agropyron*) all show the same result. Seed may be set, but it can only germinate and produce a mature plant if given artificial treatment. Brink, Cooper, and Ausherman (1944) have obtained a hybrid between *Hordeum jubatum* and *Secale cercale* but only by rearing the plant from an artificially cultivated embryo. Ragulin (1946) crossed *Elymus giganteus* with *Triticum durum* and also with *T. timopheevi* and other hexaploid wheats. Seed was set but would only germinate on artificial media. Again Pissarev and

Vinogradova (1944) produced a *Triticum vulgare*×*Elymus arenarius* hybrid, but only when each parent plant had developed from an embryo grafted on to the endosperm of the foreign genus. It is probable, as suggested by Brink et al. (1944), that some interaction between endosperm and embryo is responsible for the failure of development of the hybrid seed.

Thus under natural conditions the genera of the Elymineae would be effectively isolated from the genera of the Triticineae except *Agropyron*, and should the members of the genus *Agropyron* differentiate further so that crossing with genera of the Elymineae was not possible, the Hordeae comparium would be split, and two intersterile groups would be evolved.

Summary

1. Two natural hybrids, *Agropyron pungens*×*junceum* and *Agropyron repens*×*junceum*, are described and compared with the parental species in respect of fourteen characters.

2. It was found that the flowering times of the parental species did not overlap in 1946 and 1947, and thus hybridization was impossible.

3. The various intergeneric crosses made within the tribe Hordeae are summarized, and a rule suggested for crosses between the sub-tribes Elymineae and Triticineae.

4. A list of natural hybrids in the Hordeae is given in the Appendix.

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Appendix

A List of Natural Hybrids in the Hordeae

A. Hybrids suggested on morphological evidence, vicinity to parents, and possible sterility.

B. Hybrids proved on the evidence in A together with chromosome studies.

C. Hybrids proved on the evidence in A and B together with artificial synthesis.

Natural hybrid.	Originally described as	Authority.
A		
1. *Agropyron junceum* (L.) Beauv. × *Elymus arenarius* L. (*Triticum junceum* L. × *Hordeum arenarius* Aschers.)		Focke, 1881
2. *Agropyron pungens* R. and S. × *Elymus arenarius* L.		Hubbard, 1936
3. *Agropyron trachycaulum* (Link.) Steud.[1] × *Sitanion hystrix* (Nutt.) J.G.Sm.		Stebbins, Valencia, and Valencia, 1946*a*

[1] *A. pauciflorum* in Stebbins et al. (1946*a*) was re-identified as *A. trachycaulum* (Link.) Steud.

Natural hybrid.	Originally described as	Authority.
4. *Elymus glaucus* Buckl. × *Sitanion hystrix* (Nutt.) J.G.Sm.		Stebbins, Valencia, and Valencia, 1946*a*
5. *Agropyron trachycaulum* (Link.) Steud. × *Hordeum jubatum*	Possibly a component of *Elymus Macounii* Vasey	Ibid. 1946*b*
6. *Agropyron scirpeum* × *Hordeum maritimum*	? *Agropyron Rouxii* Gren. et Duval	Duval-Jouve
7. *Agropyron* sp. × *Hordeum nodosum*	? *Agropyron Rouxii* Gren. et Duval	Cugnac, 1937
8. *Agropyron* (*Roegneria*) *oschense* Roshev × *Hordeum turkestanicum* Nevski	*Agropyron Pavlovii* Nevski	Nevski, 1934
9. *Agropyron* (*Roegneria*) *macrourum* (Turcz.) Drob. × *Hordeum* (*Critesion*) *jubatum* L.	*Elymus chatangensis* Roshev	Ibid. 1934
10. *Agropyron repens* (L.) Beauv. × *Hordeum nodosum* L.		Vestergren, 1925
B		
1. *Agropyron pungens* (Pers.) R. and S. × *A. junceum* (L.) Beauv.	*A. acutum* R. and S.	Simonet, 1934
2. *Agropyron repens* (L.) Beauv. × *A. junceum* (L.) Beauv.		Östergren, 1940
3. *Agropyron Parishii* Scribn. and Sm. × *Sitanion jubatum* J.G.Sm.		Stebbins, Valencia, and Valencia, 1946*a*
4. *Agropyron trachycaulum* (Link.) Steud. × *Sitanion jubatum* J.G.Sm.	*Agropyron Saundersii* (Vasey) Hitchc.	Ibid. 1946*a*
5. *Agropyron trachycaulum* (Link.) Steud. × *Hordeum brachyantherum*[1]	Possibly a component of *Elymus Macounii* Vasey	Ibid. 1946*b*
6. *Elymus glaucus* Buckl. × *Sitanion jubatum* J.G.Sm.	*Sitanion Hanseni* (Scribn.) J.G.Sm.	Ibid. 1946*a*
C		
1. *Agropyron trachycaulum* (Link.) Steud. × *Elymus glaucus* Buckl.		Ibid. 1946*a*
2. *Hordeum brachyantherum* Nevski × *Elymus glaucus* Buckl.	A component of *Elymus aristatus* Merr.	Ibid. 1946*b*
3. *Elymus condensatus* Presl. × *E. triticoides* Buckl.	*E. triticoides* subsp. *condensatus* Gould	Stebbins and Walters, 1949

[1] *H. nodosum* in Stebbins et al. (1946*a*) was re-identified as *H. brachyantherum* Nevski.

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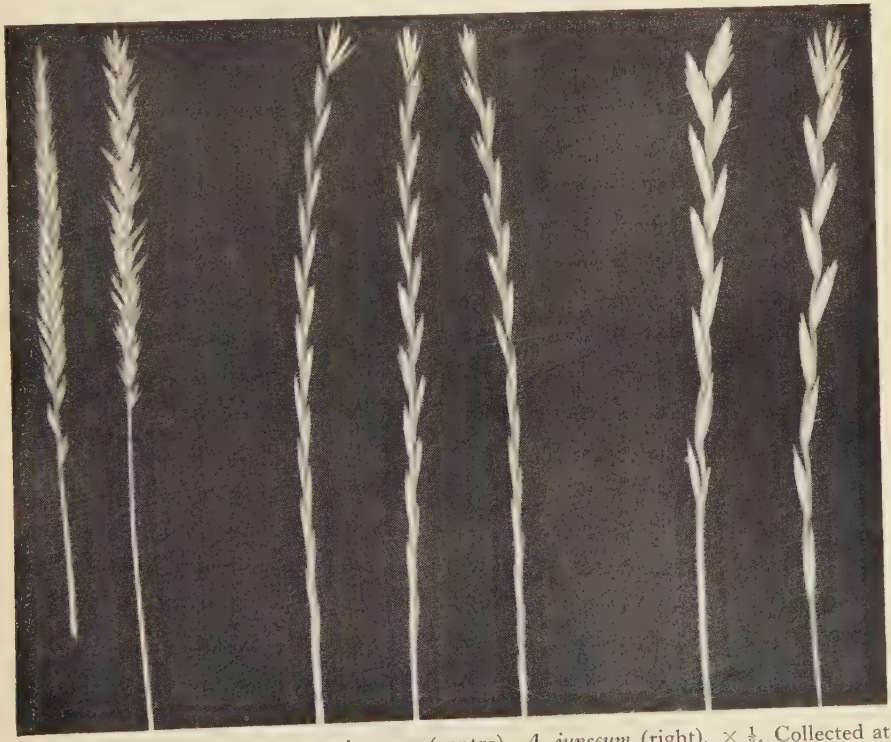
a. *A. pungens* (left), *A. pungens* × *junceum* (centre), *A. junceum* (right), × ½. Collected at Heacham, Norfolk.



b. *A. repens* (left), *A. repens* × *junceum* (centre), *A. junceum* (right), × ½. Collected at Ballywater, N. Ireland.

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